IRON AND ZINC BIOACCESSIBILITY OF FERMENTED CEREALS: LESSONS DRAWN FROM ZIMBABWEAN TRADITIONAL PORRIDGES

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“Education is the passport to the future, for tomorrow belongs to those who prepare for it today”
Malcom X.

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Thank You!!

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Summary

The goal of this PhD was to evaluate the potential of fermentation as a food based strategy to improve the bioaccessibility of iron and zinc from cereal based complementary porridges commonly consumed in Zimbabwe and Africa at large. To attain this goal, several studies were conducted to 1. understand traditional practices and utilization of cereals leading to the preparation of complementary porridges; 2. investigate the effect of household fermentation on the level of mineral binders and subsequent iron and zinc bioaccessibility; 3. determine the iron and zinc bioaccessibility of fermented cereals from different locations; 4. decipher the bacterial diversity of fermented slurries prepared from different cereals and originating from different locations; 5. understand the effect of the mineral binders phytic acid (PA), phenolic compounds (PC) and condensed tannins (CT) on iron and zinc bioaccessibility through the use of enzymes; and 6. finally to determine the potential of combining fermentation and food-to-food fortification to improve iron and zinc bioaccessibility of cereals.

In **Chapter 1**, a comprehensive literature review is given first discussing the importance of iron and zinc to human health and secondly the major modulators of iron and zinc bioavailability which include the mineral absorption inhibitors (PA, PC, CT, dietary fibers) and the mineral absorption enhancers (ascorbic acid, organic acids and meat). Having explained the importance of iron and zinc to children and hence the importance of complementary porridges, an overview of the fermented porridges normally consumed by children from Africa is given. This is followed by an appraisal on the effect of fermentation on the mineral binders from several fermented porridges and the subsequent iron and zinc bioaccessibility. Furthermore, as the microbial diversity is important in fermented products, the microbial diversity of several fermented porridges is given and the microbes with ability to metabolize PA and PC from certain porridges are highlighted. The potential of using functional starter cultures to produce cereal porridges with improved mineral bioavailability is assessed. In order to determine bioaccessibility of iron and zinc, an *in vitro* dialyzability method is used and this method and its relevance in determining iron and zinc bioaccessibility is evaluated. Additionally, an evaluation of the probable reactions that could be taking place during iron and zinc digestion is given.

Chapter 2 introduces the major sampling area which was the Ushe communal area, located in the Hwedza district of Zimbabwe. The Ushe communal area has been identified as one of the areas in Zimbabwe vulnerable to climate change and also located in a region with the highest stunting and anaemia rates. To increase resilience to climate change, one of the strategies suggested by the Soil Fertility Consortium of Southern Africa is increasing the adoption of small seeded grains such as sorghum and millets as they are drought resistant. Complementary porridges were prepared using either maize and finger millet and due to the importance of finger millet in the face of climate change,

it was chosen for this study. The processing methods of complementary porridges emerged as mainly fermentation and cooking.

After having learnt the generalized preparation process of fermented porridges, fermented finger millet porridges were prepared in several households categorized into four groups. The porridges were prepared at the household level such that they depicted the current characteristics of the porridges consumed in Ushe communal area. **Chapter 3** discusses the effect of the traditional fermentation and cooking on mineral binders and their subsequent effect on iron and zinc bioaccessibility. Bioaccessibility was defined as the proportion of minerals able to pass through a dialysis membrane of 12-14 molecular weight cut-off. The effect of fermentation on PA, PC and CT after fermentation and cooking were marginal such that there was no improvement in iron and zinc bioaccessibility. An important result from chapter 3 was the low iron and zinc contents of the finger millet and the lack of PA reduction which was not expected after fermentation. This observation was attributed to 1. the location which is known to have non-fertile soils giving rise to low iron and zinc contents and 2. the type of fermentation and cereal which probably did not enable the proliferation of microbes with phytase activity. This hypothesis led to the investigation in **chapter 4** whereby different cereal grains used in the preparation of porridges countrywide were collected from five different locations and a laboratory fermentation was performed. Mineral contents and mineral inhibitor contents differed with regards to both location and type of cereal. Fermentation caused a reduction of PA by 20-80% and caused increases in soluble PC counteracted by a general decrease in bound PC. In terms of the mineral bioaccessibility, iron bioaccessibility ranged from 2.77-26.1% while zinc bioaccessibility ranged from 0.45-12.8%. Cereals from two locations were contaminated by soil iron and it was concluded that communities from these locations could be less vulnerable to iron deficiency than people from the three other locations. It was also shown that pearl millet could be a target for studies aimed at improving iron and zinc bioaccessibility as it contained more iron and zinc. In terms of zinc, it was concluded that risk of zinc deficiency could be higher in all locations based on the low zinc content and low zinc bioaccessibility.

In **chapter 5**, a high throughput sequencing methodology was used to decipher the bacterial diversity of the fermented slurries studied in chapter 3 and 4. Bacterial communities of both household and laboratory fermented slurries were composed of bacteria from the genera *Lactococcus*, *Leuconostoc*, *Weissella* and *Enterococcus* and some Proteobacteria belonging to unclassified *Enterobacteriaceae*, and the genera *Aeromonas* and *Pseudomonas*. Unclassified *Enterobacteriaceae* were dominantly present in red sorghum and this was likely a result of the failure of lactic acid bacteria in particular *Lactococcu*s which were dominant in other fermented slurries, to metabolize the PC and CT in red sorghum. The major difference in the bacterial diversity of the fermented cereals was mainly attributed

to presence of PC and CT. In particular, a remarkable difference was observed between the bacterial diversity of fermented slurries of red sorghum with that of other cereals which had lower levels of PC and no CT.

After partial reduction of PA and some marginal changes on PC, fermentation could not evidently improve iron and zinc bioaccessibility. An enzymatic study was conducted in **chapter 6** to understand the magnitude of the effect of PA and PC on iron and zinc bioaccessibility of the cereals. After phytase treatment, a positive effect on zinc bioaccessibility was observed in all cereals while marginal changes in zinc bioaccessibility were observed after a combination of PA, PC and CT degrading enzymes was employed. Pertaining to iron, phytase treatment did not cause an effect on iron bioaccessibility while positive effect on iron bioaccessibility was only observed after PA, PC and CT degrading enzymes were employed. The study showed that complete dephytinization of cereals may not be necessary such that, the partial reduction of PA attained by fermentation may be sufficient. Furthermore, modulators of iron and zinc bioaccessibility in cereals probably go beyond the known mineral binders i.e. PA, PC and CT, and may involve matrix effects, preprocessing effects such as decortication, localization and speciation of iron and zinc in the different types of cereals, kinetics of release of iron and zinc during digestion and competition for complexation of minerals between soluble and insoluble mineral binders.

It was established that complete PA reduction may not necessarily improve iron and zinc bioaccessibility, and that targeting PC as mineral binders poses challenges as the mineral binding ability of the metabolic products from PC is not known. The changes exhibited after fermentation since not enough to cause a unanimous effect on iron and zinc bioaccessibility may need to be combined with another strategy. Food-to-food fortification was done in **chapter 7** through the addition of ascorbic acid rich baobab fruit pulp (*Adansonia digitata*) and mineral rich mopane worm (*Imbrasia belina*) to fermented cereal slurries. A positive effect on both iron and zinc bioaccessibility was observed after adding baobab fruit pulp while variable effects were observed after adding mopane worm. Mopane worm however, improved the iron and zinc contents of the fermented cereals such that mopane worm enriched cereals could contribute more iron and zinc to the recommended dietary allowance of children aged 1-3 years than the baobab fruit pulp enriched cereals. It was concluded that both baobab fruit pulp and mopane worm could have a potential to improve iron and zinc nutrition in developing countries.

Finally in **chapter 8**, the general discussion, future perspectives and conclusions are given. The reliability of the dialyzability assay to predict iron and zinc bioavailability is discussed and it was established that dialyzability may be a better predictor of zinc than of iron bioavailability. Fermentation

was considered to be a useful process in terms of partial PA reduction which is needed and also reduction of pH which provides a buffering capacity during gastric digestion thereby enhancing the solubilization of iron and zinc. The synergistic effect of fermentation and food-to-food fortification was recognized and it was recommended for such strategies to also consider other minerals such as selenium and calcium whose deficiencies are also highly prevalent in developing countries.

Samenvatting

Het beoogde doel van deze doctoraatsthesis was het potentieel van fermentatie na te gaan als een mogelijke strategie om de biobeschikbaarheid van ijzer en zink in graangebaseerde aanvullende pappen, zoals voornamelijk geconsumeerd in Zimbabwe en in Afrika in het algemeen, te verhogen. Om dit doel te kunnen bereiken werden volgende subdoelstellingen onderzocht: 1. Het beschrijven en oplijsten van de traditionele gebruiken in Zimbabwe om aanvullende graanpappen te bereiden; 2. de impact van fermentatie bij het bereiden van aanvullende graanpappen, zoals uitgevoerd binnen de lokale gemeenschappen, op de mineraalinhibitoren en dus op de biotoegankelijkheid van ijzer en zink te kennen; 3. inzicht te krijgen in de biotoegankelijkheid van ijzer en zink van graangebaseerde, aanvullende, gefermenteerde pappen afkomstig van verschillende locaties binnen Zimbabwe; 4. kennis verwerven omtrent de microbiële gemeenschap in de gefermenteerde slurries, bereid met verschillende granen afkomstig van verschillende locaties binnen Zimbabwe; 5. het beter begrijpen van de effecten van mineraalinhibitoren zoals fytinezuur (PA), fenolische componenten (PC) en gecondenseerde tannines (CT) op de ijzer en zink biotoegankelijkheid, en hoe enzymen, welke de mineraalinhibitoren degraderen, een verhoogde biotoegankelijkheid aan mineralen kan teweegbrengen; 6) het potentieel bepalen van een gecombineerde behandeling namelijk fermentatie van de granen aangevuld met food-to-food aanrijking om de ijzer en zink biotoegankelijkheid in de aanvullende graanpappen te verhogen.

De doctoraatsthesis is opgedeeld in verschillende hoofdstukken zoals hieronder verder beschreven. **Hoofdstuk 1**, welke een uitgebreide literatuurstudie is, behandelt in eerste instantie het belang van ijzer en zink in relatie tot gezondheid. Vervolgens worden de componenten besproken welke een invloed hebben op de ijzer en zink biobeschikbaarheid, zoals inhibitoren (bv. PA, PC, CT en voedingsvezel) als stimulantia (bv. vitamine C, organische zuren en vlees). Nadat het belang van ijzer en zink binnen de voeding voor kinderen wordt uitgelegd, en dus het belang van aanvullende graanpappen verduidelijkt is, wordt een overzicht gegeven van de aanvullende gefermenteerde graanpappen in Afrika welke deel uitmaken van de voeding bij kinderen. Een inschatting wordt gemaakt van het effect van fermentatie bij deze pappen op de mineraalinhibitoren, en dus op de ijzer- en zinkbiotoegankelijkheid. De beschikbare kennis rond de microbiële diversiteit van deze gefermenteerde graanpappen in Afrika wordt samengevat, en mogelijke linken tussen de aanwezige micro-organismen en hun capaciteit om PC of PA te metaboliseren wordt belicht. Tot slot wordt het potentieel van functionele starterculturen besproken, welke kunnen bijdragen tot het produceren van gefermenteerde graanpappen met een verbeterde mineralenbiotoegankelijkheid. Mineralenbiotoegankelijkheid kan bepaald worden via verschillende *in vitro* methoden. De relevantie en beperkingen van deze methoden worden als laatste kort toegelicht.

In **Hoofdstuk 2** wordt meer informatie gegeven rond Ushe communal area, gelegen in Hwedza district in Zimbabwe, de regio waar een belangrijk deel van de monsters voor deze doctoraatsthesis genomen werden. Ushe communal area werd geïdentificeerd als één van de gebieden in Zimbabwe gevoelig aan klimaatsveranderingen. Deze regio is ook één van de regio's met de hoogste aantallen dwerggroei en anemie. Om de veerkracht voor klimaatveranderingen te verhogen, is één van de strategieën, zoals voorgesteld door Soil Fertility Consortium van Zuid-Afrika, het beter aanvaarden van kleine graanzaden zoals gierst en sorghum, onder meer door de droogresistentie van deze granen. In dit onderzoek werd er toegespitst op aanvullende pappen bereid met vingergierst of mais, en omwille van de belangrijkheid van vingergierst in relatie tot klimaatveranderingen, werd vingergierst gekozen om mee verder te werken in deze studie. Uit de enquêtes werd duidelijk dat fermentatie en koken de meest voorkomende procesmethodes bij de bereiding van aanvullende graanpappen was. Nu de meest gebruikte bereidingsmethoden voor gefermenteerde pappen gekend zijn, werden gefermenteerde vingergierst pappen bereid door 4 groepen van verschillende gezinnen. De pappen werden bereid zoals gebruikelijk uitgevoerd door de gezinnen in Ushe communal area. **Hoofdstuk 3** behandelt dan ook het effect van deze traditioneel uitgevoerde fermentaties en kookprocessen op de profielen van de oplosbare en gebonden fenolische componenten. Biotoegankelijkheid werd gedefinieerd als het aandeel mineralen dat een dialysemembraan (maximale moleculair gewicht 12-14 kDa) kon passeren. Het effect van fermentatie op PA, PC en CT na fermentatie en koken was minimaal, zodat geen verhoging in de ijzer- en zinkbiotoegankelijkheid werd waargenomen.

Een belangrijk resultaat bekomen in hoofdstuk 3 was de lage gehaltes aan ijzer en zink aanwezig in vingergierst, en het feit dat PA niet daalde na fermentatie. Deze resultaten waren te wijten aan i) de locatie waar vingergierst groeide, namelijk deze locatie is gekend voor zijn niet-vruchtbare bodems, welke resulteren in lage ijzer- en zinkgehaltes; 2) het type fermentatie en graan, welke waarschijnlijk de groei van micro-organismen met fytase-activiteit niet toelieten. Deze hypotheses leiden tot het, experiment zoals beschreven in **hoofdstuk 4.** In dit onderzoek werden verschillende granen gebruikt voor de bereiding van pappen, waarbij de granen bemonsterd werden op verschillende locaties in Zimbabwe. De fermentaties werden onder gecontroleerde omstandigheden in het labo uitgevoerd. Het type graan, alsook de locatie waar het graan bemonsterd werd, bepaalde het gehalte aan mineralen alsook aan mineraalinhibitoren. Fermentaties konden een daling in PA van 20-80% teweegbrengen. Dit zorgde voor een toename in oplosbare PC, en dus een daling in gebonden PC. De ijzerbiotoegankelijkheid varieerde van 2.77 tot 26.1%, terwijl voor zink de biotoegankelijkheid varieerde tussen 0.45 en 12.8%. Granen van 2 locaties waren verontreinigd met extrinsiek ijzer. Gezinnen van deze locaties waren dus minder gevoelig aan ijzerdeficiëntie dan gezinnen die granen van de andere plaatsen consumeerden. Er kon ook aangetoond worden dat parelgierst belangrijk kon

zijn voor verdere studies die ijzer en zink biotoegankelijkheid willen verbeteren, omwille van het hogere ijzer en zink gehalte aanwezig in parelgierst. De kans op zinkdeficiëntie was op alle plaatsen groter dan deze op ijzerdeficiëntie omwille van de lage zinkgehaltes en lage zinkbiotoegankelijkheid.

In **hoofdstuk 5** werd de bacteriële diversiteit in de gefermenteerde slurries, zoals beschreven in hoofdstuk 3 en 4, ontrafeld door gebruik te maken van 16S rRNA amplicon sequencing. De bacteriële consortia van alle gefermenteerde slurries bestonden voornamelijk uit *Lactococcus*, *Leuconostoc*, *Weissella* en *Enterococcus* genera, en bij sommige kwamen ook Proteobacteria zoals niet-geklassificeerde *Enterobacteriaceae, Aeromonas* en *Pseudomonas* voor. Niet-geklasseerde *Enterobacteriaceae* waren belangrijk in rode sorghum, waarschijnlijk omwille van de onmogelijkheid van melkzuurbacteriën, in het bijzonder *Lactococcus* welke dominant is in andere gefermenteerde slurries, om zich aan te passen in een omgeving hoog in CT en PC. De variatie in bacteriële diversiteit was hoofdzakelijk toe te schrijven aan de aanwezigheid van CT en PC. Aangezien zowel het gehalte aan PC als CT slechts minimaal gedaald was na fermentatie, kon er geen éénduidige relatie tussen de bacteriële gemeenschappen en ijzer en zink biotoegankelijkheid vastgesteld worden.

Na de kleine afname in PA en minimale veranderingen in PC, kon er niet besloten worden dat fermentatie ontegensprekelijk aanleiding heeft tot een verhoogde ijzer en zink biotoegankelijkheid. Daarom werd een enzymatische studie uitgevoerd, zoals beschreven in **hoofdstuk 6**, om de impact van PA en PC op de ijzer en zink biotoegankelijkheid te begrijpen. Na een fytase behandeling werd een positief effect op de zink biotoegankelijkheid waargenomen bij alle granen, terwijl enkel minimale verschillen konden opgemeten worden op de zink biotoegankelijkheid na een behandeling met PA, PC en CT afbrekende enzymen. Wat betreft ijzer, kon er geen effect van een fytasebehandeling op de ijzerbiotoegankelijkheid waargenomen worden, terwijl een positief effect op de ijzerbiotoegankelijkheid gemeten werd na behandeling met PA, CT en PC degraderende enzymen. Deze studie toonde aan dat volledige defytinisatie van de granen niet absoluut noodzakelijk was, maar dat een gedeeltelijke reductie in PA, zoals bereikt na fermentatie voldoende kon zijn. Naast de algemeen gekende inhibitoren van ijzer en zink biotoegankelijkheid nl. PA, PC en CT, kunnen ook matrixeffecten belangrijk zijn. Voorbeelden hiervan zijn de associatie tussen voedingsvezel en mineraalinhibitoren, voorbehandelingseffecten zoals onthullen, lokalisatie en speciatie van ijzer en zink in het graan, kinetiek waarmee ijzer en zink vrijgesteld worden gedurende de vertering, competitie voor het complexeren van de mineralen tussen de oplosbare en onoplosbare mineralenbinders.

Uit de voorgaande studies kon afgeleid worden dat een volledige reductie van PA niet noodzakelijk leidde tot een verhoogde ijzer en zink biotoegankelijkheid. Ook werd duidelijk dat de impact van

mogelijke degradatie- of conversieproducten van PC op de mineralen biotoegankelijkheid niet gekend is. De veranderingen die ontstaan na fermentatie zijn niet voldoende om éénduidige effecten te veroorzaken. Het is dus noodzakelijk om fermentatie te combineren met andere technieken om een verhoogde ijzer- en zinkbiobeschikbaarheid te bekomen. Daarom werd de impact van food-to-food aanrijking (**hoofdstuk 7**) bekeken, door toevoeging van ascorbinezuurrijke baobab fruit pulp (*Adansonia digitata*) en mineraalrijke mopane worm (*Imbrasia belina*) aan de gefermenteerde graanslurries. Een positief effect op zowel ijzer als zink biotoegankelijkheid werd waargenomen na toevoegen van baobab fruit pulp, terwijl wisselende effecten waargenomen werd na toevoegen van mopane worm. Mopane worm leidde tot hogere ijzer- en zinkgehaltes in de gefermenteerde graanslurries. Dit uitte zich in het feit dat mopane worm aangerijkte granen aanleiding gaven tot hogere ijzer en zink gehaltes, welke beter de dagelijkse aanbevolen behoeftes van kinderen tussen 1-3 jaar voldoen dan de baobab fruit pulp aangerijkte granen. Er werd besloten dat zowel baobab fruit pulp als mopane worm potentieel hebben om ijzer en zink voedingsbehoeften in ontwikkelingslanden te verbeteren.

Tot slot wordt een algemene discussie, toekomstperspectieven en besluit gegeven in **hoofdstuk 8**. De betrouwbaarheid van de dialyse-assay om ijzer en zink biotoegankelijkheid te beschrijven werd besproken. Algemeen kon er afgeleid worden dat de dialyse-assay een betere voorspeller is voor zink dan voor ijzer biobeschikbaarheid. Fermentatie wordt beschouwd als een bruikbaar proces in termen van een gedeeltelijke maar noodzakelijke reductie in PA, en resulteert ook in een pH-daling, welke een bufferende effect geeft gedurende de maagvertering, en dus een verbeterde oplosbaarheid van ijzer en zink. De synergetische effecten van fermentatie en food-to-food aanrijking werd aangetoond. Het werd aanbevolen dat voor dergelijke strategieën ook andere mineralen zal selenium en calcium in beschouwing moeten genomen worden aangezien deze deficiënties ook heel sterk voorkomen in ontwikkelingslanden.

List of Abbreviations

BEC: baobab fruit pulp enriched cereal fermented slurries

BFS: backslopped fermented slurry

BFP: porridge from BFS

BFP_p: porridge from BFS with peanut butter

CE: catechin equivalents

CT: condensed tannins

D: dialyzable

dm: dry matter

GAE: gallic acid equivalents

GDP: gross domestic product

ICP-OES: Inductively coupled plasma optical emission spectrometry

LAB: lactic acid bacteria

MEC: mopane worm enriched cereal fermented slurries

NEC: non-enriched cereal fermented slurries

NET: non-enzyme treated

P: pellet

PA: phytic acid

PC: phenolic compounds

P+L+T: phytase+laccase+tannase

RDA: recommended dietary allowance

RV1: red variety 1

RV2: red variety 2

SFS: spontaneously fermented slurry

SFP: porridge from SFS

SFP_p: porridge from SFS with peanut butter

SND: soluble non dialyzable

WV1: white variety 1

WV2: white variety 2

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Introduction

"Hidden hunger" is a global problem caused by micronutrient deficiencies and affects more than 2 billion people worldwide (Akseer et al., 2017; Global Nutrition Report, 2017; Muthayya et al., 2013). Micronutrient deficiencies although not overtly visible in vulnerable populations, have devastating negative consequences on health, economic productivity and mental development and are known to reduce the gross domestic product (GDP) of developing countries by 2-5% and account for about 7% of global disease burden (Gregory et al., 2017; Muthayya et al., 2013; Stein, 2014). The most widespread micronutrient deficiencies globally are those of iron, zinc, iodine, selenium, calcium and vitamin A (Joy et al., 2014). Iron and zinc deficiencies alone are ranked as the 16th and 40th leading risk factors underlying global disease burden (Feigin, 2016). Zinc deficiency is estimated to be responsible for approximately 4% of child deaths and global disability-adjusted life years (Black et al., 2013) whilst iron deficiency is the largest contributor to global disability-adjusted life years caused by micronutrient deficiencies (Akseer et al., 2017).

Mineral deficiencies of iron and zinc are high in developing countries particularly sub-Saharan Africa where diets are based on monotonous cereal staples which are high in mineral absorption inhibitors such as phytic acid (PA), phenolic compounds (PC) including condensed tannins (CT) and to some extent, dietary fibers, and low in mineral absorption enhancers such as ascorbic acid (Gabaza et al., 2017a; Gibson et al., 2010). Mitigation strategies to reduce iron and zinc deficiencies thus entail increasing mineral contents in cereals, reducing mineral binders and increasing mineral enhancers through approaches like fortification, biofortication, supplementation and food based strategies (Gregory et al., 2017; Saini et al., 2016). Fortification, biofortification and supplementation have been successful in many circumstances but still pose challenges in many developing countries because of their requirement for high initial investment and dependence on good infrastructure to aid in distribution, stable political policies and consumer compliance (Cockell, 2007; White and Broadley, 2005). Food based strategies on the other hand, do not require external support, may be more sustainable, economically feasible and culturally acceptable as they involve the use of traditional knowledge and already available resources that are easily accessible to even the poorest communities (Gibson et al., 2006; Tontisirin et al., 2002). Some of these strategies include processing methods such as soaking, decortication, germination, fermentation and cooking which have been found to have an effect on mineral binders and positively influence mineral bioavailability (Gibson et al., 2006). In addition, food-to-food fortification which involves the identification of locally available food sources with high mineral content or absorption enhancers is crucial to alleviating mineral deficiencies in developing countries (Cercamondi et al., 2014a; Lung'aho and Glahn, 2009).

Children below the age of 5 years have high requirements of iron and zinc as such are amongst the mostly affected age group (Muthayya et al., 2013). Long term negative consequences of iron and zinc deficiencies on childhood health can be irreversible and coupled with the fact that childhood nutrition programs are more cost effective than adult nutrition programs (Bevis, 2015), it is imperative for strategies to improve iron and zinc nutrition to target complementary porridges. Many African complementary porridges are based on maize, sorghum and millets and are normally spontaneously fermented to produce thin gruels or porridges (Gabaza et al., 2017a; Motarjemi, 2002). The fermentation of the cereals to produce complementary porridges is characterized by the proliferation of lactic acid bacteria (LAB) and yeasts some of which can metabolize PA, PC and CT (Greppi et al., 2015; Songre-Ouattara et al., 2008; Svensson et al., 2010). Moreover, the production of organic acids leading to a low pH environment after fermentation elicits the reduction of Fe^{3+} to Fe^{2+} which is more bioavailable (Gibson et al., 2006). The reduction of PA, PC and CT coupled with the low pH environment attained after fermentation could thus result in improved iron and zinc bioavailability of African cereal based complementary porridges.

The goal of this PhD is therefore to determine if fermentation, as a food based strategy, is sufficient to improve the iron and zinc bioaccessibility of fermented complementary porridges consumed in Zimbabwe. The objectives of this PhD are the following:

1. To gain some insight in the usage of cereals with particular reference to complementary feeding in one typical rural setting of Zimbabwe and subsequently determine if changes occurring to mineral binders after fermentation lead to improved iron and zinc bioaccessibility in a typical fermented cereal porridge **(Chapters 2 and 3)**
2. To investigate whether iron and zinc bioaccessibility is dependent on the origin of the fermented porridge and/or the type of cereal **(Chapter 4)**
3. To decipher the bacterial communities of fermented cereals differing in type of fermentation, cereal matrix and origin of cereals **(Chapter 5)**
4. To investigate the relative magnitude of the effects of PA, PC, and CT on iron and zinc bioaccessibility in cereals **(Chapter 6)**
5. To determine the potential of food-to-food fortification on the iron and zinc bioaccessibility of fermented cereals consumed in Zimbabwe **(Chapter 7)**

The schematic outline of this PhD thesis is presented in **Figure 1**.

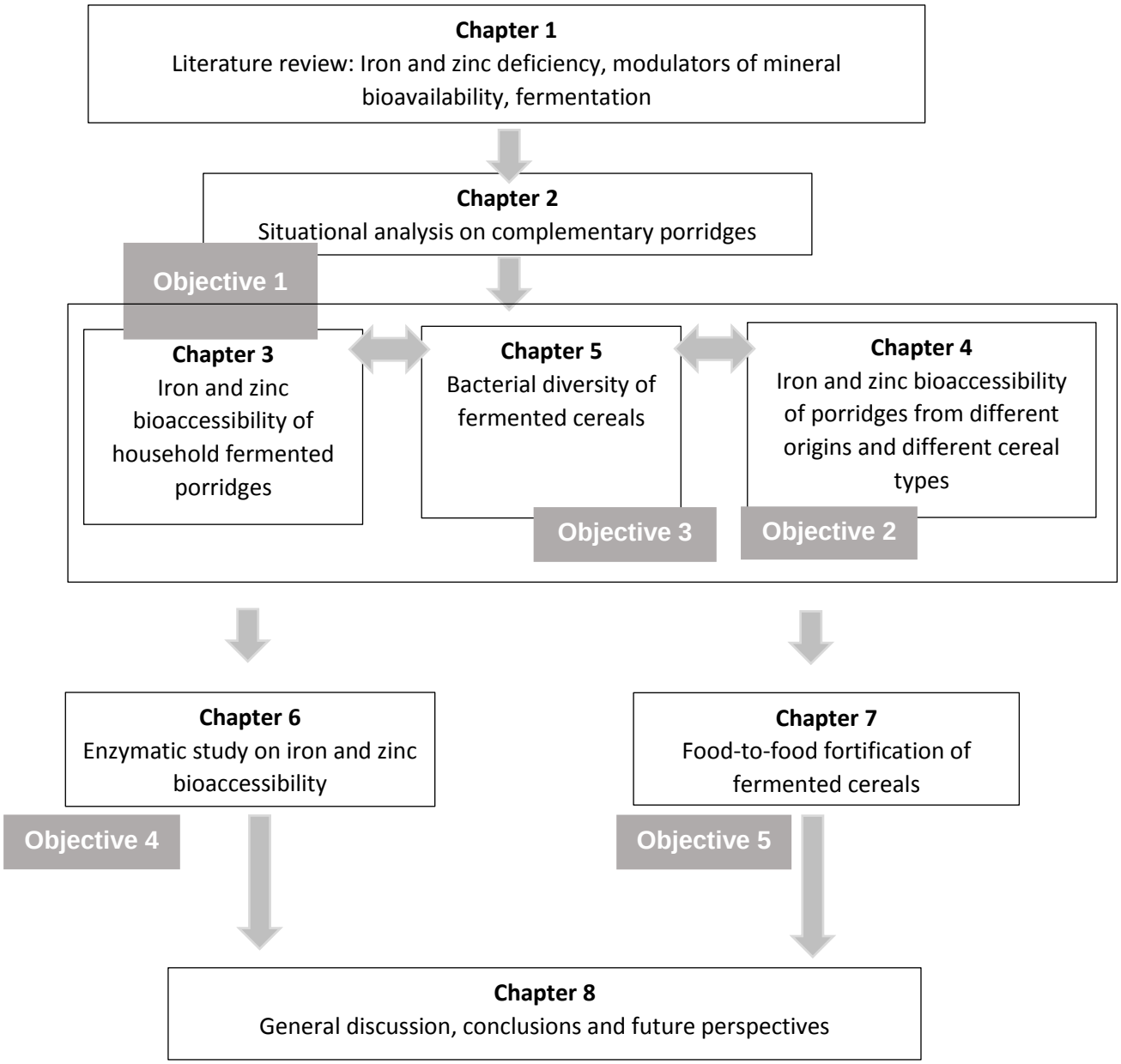


Figure 1: Schematic outline of this PhD research

Chapter 1:

Literature Review

Redrafted from:

Gabaza, M., Muchuweti, M., Vandamme, P., Raes, K., (2017). Can fermentation be used as a sustainable strategy to reduce iron and zinc binders in traditional African fermented cereal porridges or gruels? Food Reviews International 33(6), 561-586.

Chapter 1 : Literature Review

1.1 Iron

A typical 70 kg man has about 4 g of body iron and about 60% of this iron is within the oxygen transport protein haemoglobin, while about 25% is stored as readily mobilizable iron stores. The rest is bound to myoglobin in muscle tissue and in a variety of enzymes mediating in many oxidative metabolism processes and other cell functions (Abbaspour et al., 2014; Zimmermann and Hurrell, 2007). Iron is present in the diet as haem or non-haem iron. The former comprises about 40% of the iron in animal based foods while the latter accounts for the remaining 60% in animal based foods and 100% in plant based foods. This indicates that all of the dietary iron in plant based diets is existing as non-haem iron, with a much lower absorption (2-20%) compared to haem iron (15-35%) (Gibson et al., 1997). Non-haem iron is transported from the intestinal lumen to the enterocytes by the divalent metal iron transporter 1 (DMT1) which transports Fe^{2+} (Zimmermann and Hurrell, 2007). Fe^{3+} thus has to be reduced to Fe^{2+} for it to be absorbed.

Iron performs multiple roles in the human body due to its functional interconversion of oxidation states as such it is an important element to almost all living organisms. The most common role of iron is oxygen transport and storage. In addition, iron is involved in energy, protein and nucleotide metabolism, hormone synthesis and DNA replication (Abbaspour et al., 2014). Iron deficiency occurs when the physiological requirements cannot be met by iron absorption from the diet. Infants and young children have high iron requirements due to their rapid growth, followed by adolescents, women of reproductive age particularly pregnant women (Zimmermann and Hurrell, 2007). About 48% of children between the ages of 5 and 14 and 52% of pregnant women in developing countries are estimated to be anaemic according to WHO. Although anemia can occur without iron deficiency, it is used as a proxy of iron deficiency as “iron deficiency is not only the cause of anemia but where anemia is prevalent, iron deficiency is usually the most common cause”(Stoltzfus et al., 1998).

Iron deficiency manifests in many different complications that include reduced motor activity, impairment of cognitive development and increased susceptibility to upper respiratory tract infections due to weakened immune system in infants and children (Gregory et al., 2017; Zimmermann and Hurrell, 2007). In adults, iron deficiency can cause reduced work capacity and learning ability and adverse pregnancy outcomes that include increased risk of sepsis, maternal mortality, perinatal mortality, and low birth weight (Abbaspour et al., 2014). Iron deficiency thus has a devastating effect on the productivity of a nation and leads to increased economic and health costs. According to Stein (2014), iron deficiency anaemia led to the loss of 45 million disability-adjusted life years in 2010 alone.

Although non-haem iron is lowly bioavailable, its quantity is higher in many foods than haem iron such that it contributes to iron nutrition more than haem iron (Abbaspour et al., 2014). Iron requirements are thus classified based on the bioavailability of the diet which depends on the level of absorption enhancers and inhibitors as shown in **Table 1.1**.

Table 1.1: Selected recommended dietary requirements for iron by estimated dietary iron bioavailability

Bioavailability	Children (1-3 years)	Children (4-6 years)	Women (19-50 years)	Men (19-50 years)
15%	3.9	4.2	19.6	9.1
10%	5.8	6.3	29.4	13.7
5%	11.6	12.6	58.8	27.4

Numbers are mg/day. Recommended daily intake depends on the bioavailability of the diet: diet rich in vitamin C and animal protein=15%; diet rich in cereals and low in animal protein but rich in vitamin C=10%; diet rich in cereals and poor in both animal protein and vitamin C=5%. Age groups shown have high iron requirements. Adapted from (World Health Organization, 2004; Zimmermann and Hurrell, 2007).

1.2 Zinc

Zinc is the most ubiquitous element involved in human metabolism and its amount ranges from 1.5-2.5 g in an adult human body with about 60% located in the skeletal muscle and about 30% in the bone mass (Hotz and Brown, 2004). Zinc is needed in the functioning of over 300 enzymes and participates in gene expression and in the production and degradation of carbohydrates, proteins, lipids and nucleic acids (Gibson, 2012; Hotz and Brown, 2004). Data on the prevalence of zinc deficiency is much more scarce, but it is now known that zinc deficiency is as important as iron deficiency (Graham et al., 2000) and estimated by WHO to be responsible for 16% of worldwide lower respiratory tract infections, 18% of malaria and 10% of diarrhoea in Africa. There is no consensus yet concerning how to estimate the prevalence of zinc deficiency due to the limitations that are posed by all methods but currently, three indicators of measuring population zinc deficiency are recommended i.e. use of plasma (serum) zinc concentrations of a population, zinc intake data calculated using FAO food balance sheets and food composition tables and stunting prevalence in the population (Engle-Stone et al., 2014; Gibson, 2012; Wessells and Brown, 2012).

Physiological requirements for zinc are high for infants, adolescents, pregnant and lactating women (Gibson, 2012). Deficiency of zinc in children is known to manifest as reduced growth and development, increased respiratory tract infections, impaired immunity and increased mortality and

morbidity (Gibson, 2012; Hotz and Brown, 2004). Unlike iron, deficiency of zinc in populations is mainly a result of dietary factors particularly the consumption of foods with low zinc content and low zinc bioavailability or a combination of the two. This can be induced by consumption of monotonous plant products without consumption of flesh foods such as meat, fish and poultry which are good sources of zinc (Gibson, 2012). The recommended dietary requirements for zinc are thus also classified according to the type of diet as shown in **Table 1.2**.

Table 1.2: Selected recommended dietary requirements for zinc by estimated dietary zinc bioavailability

Bioavailability	Children (1-3 years)	Children (4-6 years)	Adolescent females (10-18 years)	Adolescent Men (10-18 years)
50%	2.4	2.9	4.3	5.1
30%	4.1	4.8	7.2	8.6
15%	8.3	9.6	14.4	17.1

Numbers are mg/day. Recommended daily intake depends on the bioavailability of the diet: refined diets high in animal protein and low in PA (PA/Zn<5)=50%; mixed diet, PA/Zn between 5 and 15=30%; diet rich in whole grain non-fermented and non-germinated cereals, poor in animal protein and high in PA (PA/Zn>15)=5%. Age groups shown have high zinc requirements. Adapted from (World Health Organization, 2004).

1.3 Iron and Zinc binders

1.3.1 Phytic acid

Phytic acid (myo-inositol hexaphosphate IP6) (PA) and phytate (PA associated with salts e.g. magnesium, calcium and potassium) are the principal storage forms of phosphorus abundantly present in plants. PA binds minerals, especially zinc, iron, calcium, manganese, and also proteins, forming complexes of variable solubility at physiological pH, most of which are not absorbed by the human body (Coulibaly et al., 2011; Greiner et al., 2006) due to the absence of intestinal phytate degrading enzymes (Gibson et al., 2010). PA is also known to complex endogenously secreted minerals such as zinc rendering them also inaccessible for reabsorption (Sandstrom, 1997). The antinutritional properties of phytic acid against mineral absorption is well documented and has been confirmed by several *in vivo* studies (Brnić et al., 2014; Egli et al., 2004; Hurrell et al., 2003).

The binding effect of phytic acid to minerals is dependent on the mineral in question. In terms of the type of mineral, phytic acid forms soluble salts with the alkali metals sodium and potassium, but it

forms salts of various insolubility with alkali earth metals and transition metals (Coulibaly et al., 2011). According to Engle-Stone et al. (2005), PA bound to iron in cereal grains such as wheat exists as monoferric phytate which is soluble but of low bioavailability. They attributed the low bioavailability of the iron to the steric hindrances caused by the high molecular weight PA at the brush border surface. Phytic acid is also said to form complexes with higher stability constants with trivalent metal ions than with divalent metal ions (Torres et al., 2008). The presence of two minerals simultaneously with phytate may produce a more enhanced complexation between the minerals and PA. This is widely reported for zinc and calcium whereby the formation of a calcium-zinc-phytate complex is stronger than when it is just with either of the minerals (Coulibaly et al., 2011; Greiner et al., 2006). Besides the type of mineral ion, the stability and solubility of PA mineral complexes seem also to be dependent on the pH, the phytate: cation molar ratio, the nature of the PA i.e. lower inositol phosphates vs. higher inositol phosphates and their different isomeric forms, as well as on the presence of other compounds. Bretti et al. (2012) found that phytic acid shows selectivity in mineral binding based on the pH of the medium.

The inhibitory effect of PA to iron and zinc absorption is known to follow a dose dependent relationship (Hallberg et al., 1989; Nävert and Sandström, 1985). The inhibitory effect of phytate seems to be stronger for iron compared to zinc, and results in a significant negative effect on iron absorption when 2-10 mg phytate phosphorus is present, while for zinc, negative effects are seen from contents of 50 mg phytate phosphorus onwards (Lönnerdal, 2002). Therefore, molar ratios are used to predict mineral absorptions. The recommended ratios for iron is PA/Fe < 1 (Hurrell, 2004) while the PA/Zn ratio has been estimated according to PA content and type of diet consumed. Ratio of 5-18 has been defined for mixed or refined vegetarian diets with absorption of 27% (adult men), 35% (adult women), 31% (children) and PA/Zn > 18 for unrefined cereal based diets with estimated absorption of 19% (adult men), 26% (adult women) and 23% (children) (Hotz and Brown, 2004). Bioavailability of zinc can thus be split into three categories i.e. PA/Zn < 5 for high bioavailability (50%), PA/Zn between 5-18 for moderate bioavailability (30%) and PA/Zn > 18 for low bioavailability (15%) (World Health Organization, 2004). However these ratios are used for adults, while no evidence exists that they are also appropriate for young children (Gibson et al., 2010). Sandstrom et al. (1983) already suggested that the absorption of zinc was more effected by phytate at a younger age. Also these bioavailability ratios can only be used when phytate is the only inhibitor present, which is not the case with many food products. Indeed, several studies have proved that the inhibitory effect of PA is not only dose dependent but also highly dependent on the food matrix in question. For example, the inhibitory effects of PA can be reduced in the presence of ascorbic acid and meat but not in the presence of phenolic compounds (Cercamondi et al., 2014b; Engle-Stone et al., 2005).

After hydrolysis of phytic acid, lower inositol phosphates of different isomeric forms are produced due to the differences of the positional hydrolysis of the phytase enzymes. Phytic acid with less than five phosphate groups (IP1-IP5) does not impact on zinc absorption while those with less than three phosphate groups (IP1-IP3) do not impact on iron absorption (Lönnerdal et al., 1989; Sandberg et al., 1999). Most researchers are measuring IP5 and IP6 as these are the most abundant in foods and generally accepted as the most inhibitory against mineral absorption. According to Sandberg et al. (1999), the isomer inositol 1,2,3 phosphate forms strong complexes with Fe^{3+} at pH 7.0 but Skogland et al., (1999) failed to find a difference in mineral binding in Caco-2-cells when different isomers of phytate were used. In general, it is suggested that weaker and more soluble complexes are formed with lower inositol phosphates than with the higher inositol phosphates. This implies that the stability and solubility of mineral-phytate complexes is a function of the level of phosphorylation of PA (Lönnerdal et al., 1989; Sandberg et al., 1999). **Figure 1.1** shows the structure of PA and an example of its chelate.

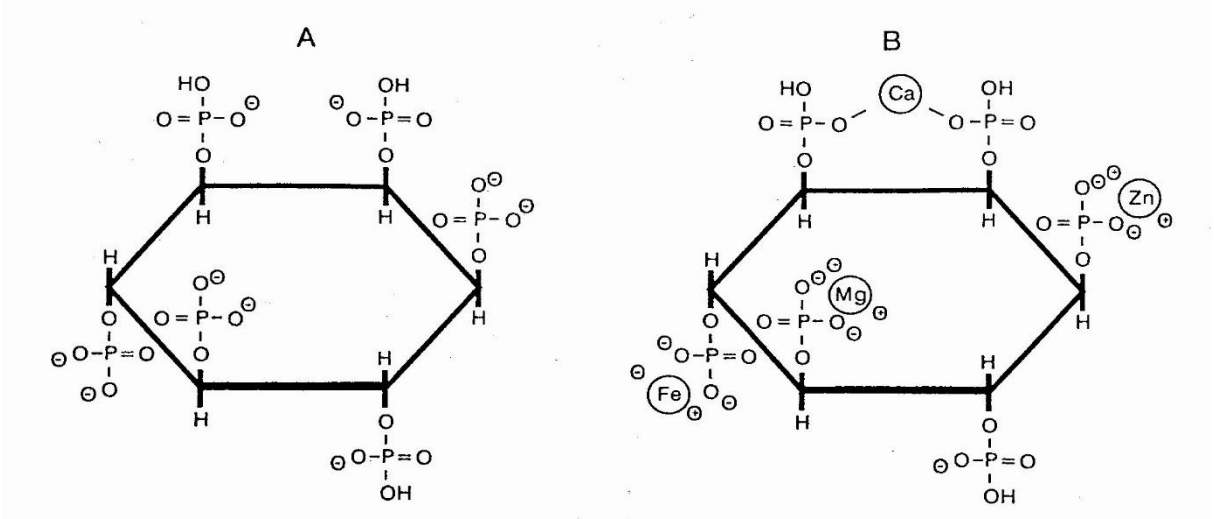


Figure 1.1: Structure of phytic acid (A) and phytic acid chelate (B)
Retrieved from: Kong et al. (2015)

1.3.2 Phenolic compounds

Phenolic compounds (PC) are a wide and extremely complex group of plant substances ranging from fairly simple molecules such as phenolic acids to highly complex polymerized compounds such as tannins. PC are ubiquitous in vegetables, fruits, legumes and cereals. In cereals, the amount of PC is usually less than 1% of the dry matter content, with the exception of some sorghum varieties which have been found to contain up to 10% PC (Bravo, 1998). PC bind and precipitate macromolecules such as carbohydrates and proteins, thereby influencing their digestibility (Bravo, 1998). Their antinutrient

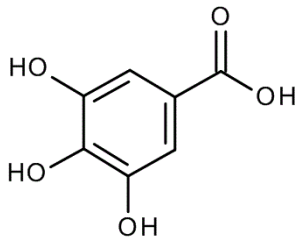
properties have also been extended to mineral bioavailability. PC have been found to chelate certain minerals through their hydroxyl groups, forming insoluble complexes which cannot be absorbed by the human body (Bravo, 1998). The complexation of PC towards iron is described to some extent, while information towards zinc is rather scarce.

The efficiency of PC to bind metals is dependent on several factors: type of metal and its redox state, structure of the PC, mineral: PC ratio and pH (Andjelkovic´ et al., 2006; Chvatalova et al., 2008; Khokhar and Owusu Apenten, 2003; Knockaert et al., 2014; Macakova et al., 2012). In general ortho-dihydroxy (catechol) or trihydroxy (galloyl) groups need to be present in the structure of the PC to be able to bind iron (Brune et al., 1991; Khokhar and Owusu Apenten, 2003) and some of the structures of PC known to have mineral binding properties are shown in **Figure 1.2**. Knockaert et al. (2014) showed that the interaction between iron and gallic acid was pH dependent and different if iron was present as Fe^{2+} or as Fe^{3+}. Phenolic acids bearing a catechol group form complexes both with Fe^{2+} and with Fe^{3+}, but the Fe^{3+} phenolic acid complexes are the most dominant (Chvatalova et al., 2008). Also Chvatalova et al. (2008) showed that some phenolic acids-iron chelates play an important role in the Fe^{2+} autooxidation. The Fe^{2+} form is the major form absorbed, such that this process could be important for increasing iron bioavailability.

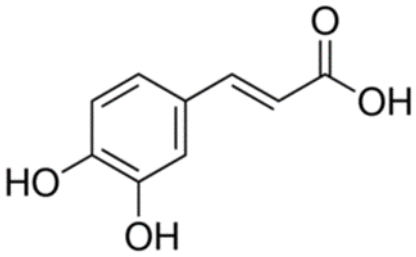
A more abundant group of PC in nature are flavonoids, characterised by their structure consisting of two aromatic rings (ring A and ring B), linked by ring C which is an oxygenated heterocycle formed by three carbon atoms (Manach et al., 2004). For iron binding, one agrees that a vicinal di-hydroxyl group is required. More in general it could be stated that the 3',4'-dihydroxygroup on ring B (catechol) has a higher binding efficiency compared to the 3',4',5'-trihydroxygroup on ring B (galloyl) or the 3',4',5'-trihydroxygroup on ring C (epicatechin gallate) (Bravo, 1998). The presence of a double bond between C2 and C3 in the C ring also influences the iron complexation efficiency (Mira et al., 2002). Mladenka et al. (2011) and Macakova et al. (2012) have clearly demonstrated the pH dependency of the iron-flavonoid complexes. Stable complexes were formed at neutral physiological pH, while at more acidic pH the ferrous chelation was less pronounced. This could be due to the dissociation in the 7-OH group which is more prone to acidic conditions compared to the 4'-OH group and 5-OH group (Macakova et al., 2012). Also the reduction of Fe^{3+} to Fe^{2+} by the flavonoids was more pronounced at acidic pH than at neutral pH (Macakova et al., 2012) which is another important feature to increase the iron bioavailability.

Tannins are large chemical structures representing a third group of phenolic compounds. Some tannins e.g. gallotannins and ellagitannins are hydrolysable and are in fact derivatives of gallic acid. Others are

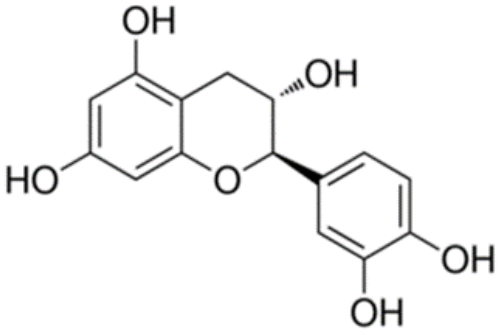
condensed forms and include mainly proanthocyanidins which are polymers of flavan-3-ols. Due to the large number of OH-groups present on the aromatic rings, they can chelate and form stable iron complexes, both with Fe^{2+} as with Fe^{3+}. These complexes are stable at physiological pH values, hampering the absorption of iron in the gastrointestinal tract (Lopes et al., 1999; Mila et al., 1996; South and Miller, 1998). Tannic acid has been observed to bind iron with relatively high affinity and proved to be a more potent inhibitor of iron than PA (South and Miller, 1998). Studies using a Caco-2-cell model system revealed that maximum inhibition of iron (90%) was observed at a molar ratio of 1:1 iron: tannic acid compared to a maximum inhibition at molar ratio of 1:10 iron: PA (Glahn and Wortley, 2002).



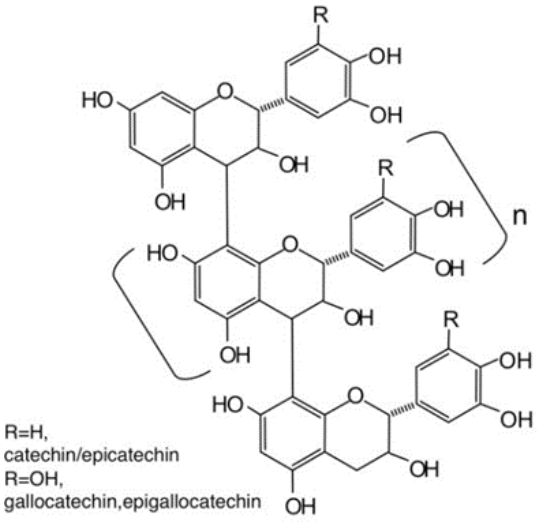
Gallic acid



Caffeic acid



Catechin



Proanthocyanidins

Figure 1.2: Structure of some phenolic compounds with di- and tri- hydroxyl groups
Retrieved from: (Andjelkovic´ et al., 2006; Schofield et al., 2001)

In vitro studies with zinc to investigate the complexation with PC are rare. Some researchers have found no effect of PC on zinc absorption (Afsana et al., 2004; Brnić et al., 2014) while others did

(Coudray et al., 1998). Coudray et al. (1998) reported that chlorogenic acid and caffeic acid reduced zinc bioavailability in rats while protocatechuic acid had no effect. Zinc has a low affinity for PC especially at acidic and neutral pH (Afsana et al., 2004; Flanagan et al., 1985; Santos-Buelga and Augustin Scalbert, 2000). PC seem to chelate iron more readily than zinc in solutions at physiological pH. It is clear that more research is needed in this area to better understand the role of PC in mineral bioavailability of food matrices. The main challenge that is presented by PC is their well-known therapeutic benefits in the human body. It is imperative that a compromise be found so that PC are not present in too high amounts to inhibit mineral absorption, but at the same time in not too low concentrations for the human body to benefit from their antioxidant activity.

1.3.3 Dietary fiber

Dietary fiber is now generally defined as oligosaccharides, polysaccharides, the (hydrophilic) derivatives and non-carbohydrate components such as lignin which cannot be digested by the human digestive enzymes to absorbable components in the upper alimentary tract (Dhingra et al., 2012; Ha et al., 2000; Thebaudin et al., 1997). Dietary fibers play a beneficial function in the human body especially in terms of disease risk reduction as such whole grains are widely encouraged to be part of a healthy diet. Nonetheless, a diet rich in dietary fiber is also related with mineral deficiencies as dietary fiber (and compounds associated with it i.e. PA and tannins) is known to bind minerals such as iron, zinc and calcium among others making them unavailable for absorption. Most studies dealing with the chelating ability of dietary fiber with minerals are from more than 20 years ago with lack of recent information providing more in detail mechanisms of action. This could be because the benefits of dietary fiber are undisputed and there is no consensus among researchers on the negative effect of dietary fiber to mineral bioavailability.

The chelating ability of dietary fiber is a result of electrostatic interaction (Debon and Tester, 2001; Torre et al., 1995) and adsorption (Lakshmi and Sumathi, 1997; Torre et al., 1995). The electrostatic interaction is because dietary fibers contain many free carboxylic and sulfonated groups and these negatively charged groups can interact with mineral ions. Nair et al. (1987) observed that higher mineral binding occurred in low methoxylated pectin than in high methoxylated pectin proving that the fiber-mineral interaction is highly electrostatic in nature. Low mineral binding was observed in cellulose and this could be because it contains few charged groups (Nair et al., 1987; Persson et al., 1987). Dietary fibers can be water soluble and water insoluble and the former has been found to be closely associated with minerals and up to 75% of total PA (Persson et al., 1991). The mineral binding ability of dietary fiber seems to be dependent on a variety of factors. The most important factor is pH

which has been studied extensively. It has been shown that mineral binding is minimal at low pH (around 4) and maximal at pH 5.8-6.5 (Lakshmi and Sumathi, 1997; Matin et al., 2013; Persson et al., 1991) which is also simulated to be the pH range for the small and large intestine. Lakshmi and Sumathi (1997) found low iron binding at pH 4.0 and the highest binding at pH 6.5. This could suggest that during fermentation mineral binding of fibers is lowered as pH is decreased.

Another important factor is the presence of other mineral binding components in the diet or the fiber matrix itself such as PA and PC. In a study that was done by Persson et al. (1991) using soluble fiber fraction from barley and rye flour, they observed that zinc binding was remarkably reduced at pH <6 after dephytinization although still some binding was observed at pH >6. This shows that PA is strongly associated with fiber fraction and its removal greatly reduces mineral binding. The remaining binding could be due to the fiber which was mentioned before that binding is highest at pH 6.5. Other workers found that in fiber fraction of some cereals, after dephytinization, *in vitro* iron binding did not change implying that fiber and possibly other components such as PC are responsible for the binding (Baye et al., 2015; Lakshmi and Sumathi, 1997; Lestienne et al., 2005b). This shows that different plant matrices will yield different results as there is considerable variation in the types of dietary fibers, levels of PA and PC in different cereals.

Conflicting results exist about the chelation of minerals by dietary fibers and this could be owing to incomparable study designs used in a lot of the studies and use of different types of dietary fibers (Baye et al., 2017). Some researchers use purified isolated dietary fibers which are not normally found in conventional food products. Results obtained from *in vitro* experiments should be interpreted carefully as these often conflict with *in vivo* experiments. Some researchers have found that mineral absorption is improved in the presence of dietary fiber (Coudray et al., 2003) while Martin et al. (2013) concluded that mineral binding by dietary fibers may not have detrimental effects on mineral bioavailability in humans. *In vitro* experiments fail to show the fermentation by bacterial enzymes of some dietary fibers that occurs in the large intestine. The degradation of some fermentable dietary fibers such as pectin can release mineral ions which can be subsequently absorbed (Coudray et al., 2003; Debon and Tester, 2001; Greger, 1999; Nair et al., 1987). Processing methods that release minerals from dietary fibers may be the only option to improve mineral nutrition as dietary fibers are essential for human health.

1.4 Iron and zinc absorption enhancers

Ascorbic acid is the most important enhancer of non-haem iron absorption but not of zinc (Gibson et al., 1997). Ascorbic acid is able to reduce Fe^{3+} to Fe^{2+} which is more soluble at the physiological pH of

the intestine and readily absorbed by the Fe^{2+} transporters (Cercamondi et al., 2014b; Engle-Stone et al., 2005). The complex formed between ascorbic acid and iron is weak such that in the absence of compounds that can bind or oxidize iron, the iron is highly bioavailable (Engle-Stone et al., 2005). Radioisotope studies in humans have shown that the enhancing effect of ascorbic acid to iron availability either in its natural or synthetic form, is dose dependent (Hallberg et al., 1992). The magnitude of this enhancing effect is dependent on the molar ratio of ascorbic acid to iron and the amount of inhibitors present (Hurrell, 2002). Molar ratio of 2:1 ascorbic acid: Fe (20 mg ascorbic acid, 3 mg iron) is needed for foods with low-medium levels of inhibitors while a molar excess of 4:1 is required for foods with high level of inhibitors (Teucher et al., 2004). The usefulness of ascorbic acid as an enhancer of iron absorption in developing countries is however limited, as ascorbic acid is an expensive additive, which is highly unstable during heat treatments and food storage. The addition of a more stable derivative of ascorbic acid such as ascorbyl palmitate to cereal based foods deserves further attention (Hurrell, 2002).

Another important enhancer of iron absorption is muscle tissue. The effect of muscle tissue on iron absorption is known as the “meat factor” or “meat effect” and the mechanism by which it improves iron bioavailability is not fully understood. Two mechanisms are generally accepted and include the effect of small sulfated glycosaminoglycan carbohydrates and also sulfated amino acids like cysteine and peptides (Baech et al., 2003; Huh et al., 2004). These compounds are able to form soluble complexes with iron and can also reduce Fe^{3+} to Fe^{2+} at low pH thereby preventing the formation of insoluble ferric hydroxide (Amaro López and Camara Martos, 2004). As such the meat factor arises from the ability of these compounds to act as both reductants and chelators (Storcksdieck and Hurrell, 2007). Dietary sulphur containing amino acids from other food sources and not only from muscle tissue are now also suggested to improve both iron and zinc bioavailability. Sulphur containing compounds from garlic and onion had a positive effect on iron and zinc bioavailability when they were added to cereals and legumes (Gautam et al., 2010a; Kumari and Platel, 2016). Other compounds that have been suggested to have a positive influence on both iron and zinc bioavailability are organic acids, β-carotene and some non-digestible carbohydrates. β-carotene has been found to have the ability to keep iron and zinc soluble in the intestinal lumen thereby preventing the inhibitory effects of PA and PC, but the exact mechanism through which this is achieved is unclear (Amaro López and Camara Martos, 2004; Garcia-Casal, 2006; Garcia-Casal et al., 1998). On the other hand, organic acids are able to form soluble complexes with iron and zinc whose bioavailability is then dependent on strength of the complex, its concentration and presence of mineral binders such as PA and CT (Amaro López and Camara Martos, 2004). A positive effect but of varying degrees on iron bioavailability of nine organic acids i.e. tartaric, malic > succinic, fumaric > citric, lactic > acetic, propionic acid was reported

(Salovaara et al., 2002). The suggested positive effect of some non-digestible carbohydrates such as fructo-oligosaccharides on the mineral absorption is related to the fact that they are not digested in the small intestine but they end up in the large intestine where they are fermented by colonic bacteria (Amaro López and Camara Martos, 2004). Their fermentation leads to the production of short chain fatty acids such as acetate, propionate and butyrate which then form soluble complexes with minerals. It has been hypothesized that these prebiotics are responsible for some phytase production in the colon, due to their stimulating effect of microbial colonic growth such that some minerals are released by hydrolysis. Increased iron colonic absorption has been reported (Yeung et al., 2005).

1.5 Overview of African fermented porridges or gruels

Fermented cereal products are very important in Africa and are often associated with low income populations. In Africa, the major cereals of importance are maize, sorghum and millets which are used to produce a diverse amount of fermented products. The products range from beverages which can be alcoholic (e.g. *tchoukoutou, mangisi*) or non-alcoholic (e.g. *maheyu, togwa, obushera*), thin porridges sometimes referred to as gruels (e.g. *ben-saalga, uji/ogi, koko, gowé, degué, kunuzaki*) and thick porridges sometimes referred to as gels or pastes (e.g. *banku, poto poto*) to bread like doughs (e.g. *injera, kenkey, hussuwa*). In some cases, intermediate products used to make a variety of end products are also common e.g. *mawé* famous in Benin. A thorough review on these African fermented cereal products and their microbial ecology can be found elsewhere (Blandino et al., 2003; Franz et al., 2014; Guyot, 2010; Guyot, 2012; Hammes et al., 2005; Nout, 2009; Soro-Yao et al., 2014a).

Of interest in this literature review are the thin porridges/gruels and beverages with a drinkable or spoonable consistency. These products are mostly used as weaning and complimentary foods for African children and improvement of their nutritive value is highly relevant. **Table 1.3** shows the list of some of the commonly consumed fermented porridges and their country of origin. These products are also known to have a dry matter content of 6–10% making them very dilute and lacking sufficient nutrients (Nout, 2009). Complementary porridges should provide sufficient energy density for children and most porridges with a dry matter content of less than 10% are not able to fulfill energy requirements for children below the age of two (Mouquet-Rivier et al., 2008; Soro-Yao et al., 2014b). **Table 1.4** shows the energy requirements for children aged between 9-23 months who rely mostly on complementary porridges for their energy intake. It is imperative that meeting energy requirements for children be the primary consideration in the preparation of complementary porridges.

Table 1.3: Overview of African cereal fermented porridges

Name of porridge	Description	Country of origin
Mahewu	Maize based non-alcoholic porridge with additions of sorghum/millet malt	Zimbabwe, South Africa
Obushera	Collective name for sorghum and/or millet based non-alcoholic beverage	Uganda
Togwa	Maize, sorghum/millet based non-alcoholic beverage	Tanzania
Ogi/uji	Maize/sorghum based porridge	Nigeria, Benin
Koko	Millet based porridge	Ghana
Ben-saalga	Pearl millet based porridge	Burkina Faso
Degué	Millet based porridge	Burkina Faso
Kunuzaki	Millet based porridge	Nigeria
Munkoyo/ Chibwantu	Maize based non-alcoholic beverage with some additions of sorghum/millet	Zambia
Gowé	Sorghum based porridge	Benin
Ting	Sorghum based porridge	Botswana
Mawé	Maize dough used as base for other foods such as *ogi*	Benin, Togo

Retrieved from: (Blandino et al., 2003; Franz et al., 2014; Guyot, 2010; Guyot, 2012; Nout, 2009; Soro-Yao et al., 2014a).

Table 1.4: Minimum dietary energy density required from complementary foods per day according to age group and level of breastmilk energy intake

	9-11 months			12-23 months		
	Low BME	Average BME	High BME	Low BME	Average BME	High BME
Total energy required (kcal/day)	858	858	858	1118	1118	1118
BME (kcal/day)	157	379	601	90	346	602
Minimum energy density (kcal/g)						
1 meal/day	2.46	1.68	0.90	2.98	2.24	1.50
2 meals/day	1.23	0.84	0.45	1.49	1.12	0.75
3 meals/day	0.82	0.56	0.30	0.99	0.75	0.50

BME: Breastmilk energy intake. Assumed functional gastric capacity (30 g/kg reference body weight) is 285 g/meal at 9-11 months and 345 g/meal at 12-23 months. Total energy requirements is based on new US longitudinal data averages plus 25% (SD). Adapted from (Dewey and Brown, 2003).

1.6 Fermentation to reduce iron and zinc binders

Fermentation is a process led by microbial activities resulting in an improvement in the quality of the final product (Hammes et al., 2005). Due to the production of acids and/or alcohol, fermented foods have an improved shelf-life, a reduced risk for contamination with pathogens, as well as improved sensory properties such as aroma, taste, color and texture. The fermentative action of specific microorganisms can remove some undesirable compounds from foods such as cyanogenic compounds, antinutritional factors (e.g. antitrypsin), endogenous toxins and mycotoxins (FAO, 1999; Nout, 1994). The antimicrobial action of the fermenting microbiota (especially lactic acid bacteria (LAB)) which prevents the proliferation of undesirable microorganisms some of which can be mycotoxin producing fungi, may be explained by three mechanisms which include the production of organic acids, competition for nutrients and the production of antagonistic compounds (Dalié et al., 2010). Another important role of fermentation is its impact on the bioavailability of some nutrients. The bioavailability of nutrients can be increased by fermentation through the conversion and/or breakdown of antinutritional factors, the production of microbial enzymes or the activation of endogenous enzymes (Hammes et al., 2005). Of particular importance in the context of the production of complementary porridges are the changes occurring to phytates and PC which have implications on iron and zinc bioavailability.

Phytases can originate from the grain itself as well as from microorganisms. The optimum conditions in which phytases function is dependent on their source for example, the optimum pH for most cereal endogenous phytases is 4.5-5.0 (Anastasio et al., 2010; Svanberg and Lorri, 1997) while pearl millet phytases operate at pH 6.0 and 24–40 ^{o}C, wheat phytates need a pH 5.0 at 55 ^{o}C and phytases isolated from *Aspergillus ficuum* operate at pH 2.5 at 37 ^{o}C (Lestienne et al., 2005a). Most cereal fermentations start at a pH of about 6 and during fermentation the pH is reduced to between 3-4 implying that the pH interval optimum for phytase activity is traversed. The mature grain phytase activity varies extensively among grains. The reported activity in millet and sorghum is up to 10-100 times lower than activities measured in wheat, rye and barley (Brinch-Pedersen et al., 2014) therefore, to degrade phytate in sorghum and millet based products, other processing strategies need to be involved to have some effect on the mineral bioavailability. In that respect the use of microorganisms in the fermentation process with a known phytase activity can be very useful. Fungi are well-known phytase producing micro-organisms and most well-known genera where phytase has been derived include *Aspergillus, Mucor, Penicillium and Rhizopus* (Gupta et al., 2013) with *A. niger* and *A. ficuum* being some of the most efficient phytase producers (Jorquera et al., 2008). However filamentous fungi are not recommended species according to the latest Qualified Presumption of Safety (QPS) list of EFSA

(2013) because they pose a risk for the production of mycotoxins. Bacteria that produce extracellular phytase include members of the genera *Bacillus* (Choi et al., 2001; Kim et al., 1998), *Enterobacter* (Yoon et al., 1996) and *Pseudomonas* (Jorquera et al., 2008) and are used for commercial phytase production. Compared to fungal phytases, bacterial phytases have a higher substrate specificity, are more resistant to proteolysis and have a greater catalytic efficiency (Jorquera et al., 2008). Some lactic acid bacteria (LAB) have been associated with phytase production but very few LAB have been found consistent in their phytase producing activity (De Angelis et al., 2003; Zamudio et al., 2001).

Some wheat sourdough LAB were tested, and those with the highest phytase activity were strains of *Lactobacillus sanfranciscensis* followed by strains of *L. fructivorans, Weissella confusa, Lactococcus lactis* and *L. alimentarius* (De Angelis et al., 2003). Recently, some phytase active LAB have been isolated from *injera* which is a traditional Ethiopian pancake i.e. *L. brevis, L. buchneri, L. casei, L. crustorum, L. fermentum, L. plantarum* and *Pediococcus pentosaceus* with *L. buchneri* and *P. pentosaceus* exhibiting the highest phytate degrading capacity (Fischer et al., 2014). Phytase active yeasts have also been isolated from sourdough, although the full potential of yeast phytases is not well studied. Extracellular phytase production has been reported for strains of *Saccharomyces, Candida, Cryptococcus, Kluveromyces, Pichia* and *Torulaspora* species (Kaur et al., 2007). Predominant yeast phytase activity in sourdough was observed in *Saccharomyces cerevisae* L1.12, *S. cerevisae* L6.06, *Candida humilis* and *Pichia kudriavezvii* (Nuobariene et al., 2012). The potential of phytase positive starter cultures was demonstrated in a study by Anastasio et al. (2010) who added phytase positive and negative starter culture to sourdough fermentation. The phytase positive starter culture consisted of *L. plantarum*, *Leuc. gelidium* and *E. faecium* isolates from pizza dough and sourdough, while the phytase negative starter culture contained other *Leuc. gelidium* and *L. plantarum* isolates. Their results showed that addition of a phytase positive starter culture caused a higher solubility of iron (98%) and zinc (89.8%) compared to the phytase negative starter culture (41% soluble iron and 60% soluble zinc). The solubility of iron and zinc also increased when phytase negative starter culture was added because of the activation of endogenous microbial phytases. Magala et al. (2015) also observed similar trends as they measured an 89% reduction of PA in samples of tarhana (made from mixture of wheat flour and yoghurt) after fermenting it with *L. sanfrancisco* CCM 7699 and an almost 100% PA degradation in cereal beverages based on rice and oats.

There has been less progress regarding the effect of fermentation on PC and tannins and the identification of microbes responsible for their conversions. Several studies demonstrated that LAB possess different types of enzymes responsible for the conversion or degradation of phenolic compounds including reductases, dehydrogenases and decarboxylases (Knockaert et al., 2012;

Rodriguez et al., 2009; Rodriquez et al., 2008; Sanchez-Patan et al., 2012; Tabasco et al., 2011). Several researchers have observed that there is a clear reduction of PC and CT after fermentation and this could be as a result of several reasons. The production of PPO by microbes could cause the degradation of PC, the low pH as a result of the fermentation could cause the exclusion of hydride ions and subsequent reordering of PC and also the scission of proanthocyanidins into flavan-3-ols which can be oxidized to quinones (Taylor and Duodu, 2015). However, none of these mechanisms have been ascertained. It is also unclear to which extent LAB can effectively degrade or convert PC during the fermentation process. Also, LAB do not possess cell-wall degrading enzymes, resulting in their inability to degrade cell-wall polymers, and release the phenolic components from the cell wall matrix. Nonetheless it is known that there are changes in the level of PC during spontaneous LAB fermentation of maize, sorghum and millet based fermented foods (Kayodé et al., 2013; Matuschek et al., 2001; Svensson et al., 2010; Towo et al., 2006) and whether or not these changes favor mineral bioavailability is still a debatable subject. Besides the pH decrease, the lactic acid fermentation can also affect the complexation between iron and phenolic compounds (Knockaert et al., 2014) and between minerals and phytate, thus indirectly affecting the bioavailability of the minerals. On the other hand, it has been demonstrated that during fermentation of high-tannin and non-tannin sorghum and millet gruels, there is a higher increase in solubility of iron in the non-tannin cereal compared to the high tannin cereal (Cercamondi et al., 2014b; Hurrell et al., 2003; Svanberg et al., 1993). Lestienne et al. (2005a) reported that after dephytinization of both non-tannin and high-tannin millet grain fractions, there was a decrease in the PA: Fe molar ratio which should result in an increase in the *in vitro* soluble iron. However, the low PA: Fe molar ratio in high tannin millet did not increase the levels of *in vitro* soluble iron. The same effect was also observed for zinc. This means that molar ratios of phytate: mineral are only relevant when no other inhibitors such as PC and dietary fiber are present (Kruger et al., 2012; Matuschek et al., 2001; Towo et al., 2006) which is not the case for many cereal matrices. Moreover, it demonstrates that optimization of the fermentation process for high tannin sorghum and millets is needed. Most African varieties of sorghum contain tannins while for millets, only finger millet is known to contain a significant amount of tannins. These varieties are preferred because they are well adapted to the African climate (Dykes and Rooney, 2006) and also preferred for food preparation because of their desirable color compared to the non-pigmented varieties. Tannin sorghums are also known to be slowly digested thus prolonging the period of satiety (Awika and Rooney, 2004). **Table 1.5** shows the micronutrients and antinutritional factors found in sorghum, pearl millet and finger millet, the two mostly consumed millets in Africa. It is imperative to note that the high iron contents of most of the grains is mainly associated with extrinsic iron specifically from soil most of which has been found to be not bioavailable (Icard-Vernière et al., 2013).

Table 1.5: Content of micronutrients and anti-nutritional factors in sorghum and millet grains

Micronutrient/Antinutrients mg/100 g dm	Sorghum	Finger millet	Pearl millet
Phytate	290-3350	417-693	354-796
Tannins[1]	0-5000	0–2117[2]	-
Total PC	43.1-300.3	149-590	190-330
Iron	4.7-25.6	2.13–5.0	3.0-18.0
Zinc	1.54–2.54	1.50-1.73	2.51-6.0
Calcium	6-53	10-398	10-80
Magnesium	140-293	76-110	180-270
Fiber	7.8-11.8	10.0-19.1	7.0-11.0

[1]CE = catechin equivalents

[2]Tannins occur in red finger millet varieties only

Retrieved from: (Abdalla et al., 1998; da Silva and Ciocca, 2005; Devi et al., 2011; Dicko et al., 2006; Dicko et al., 2002; Dykes and Rooney, 2006; Dykes et al., 2013; Eyzaguirre et al., 2006; Hama et al., 2011; Hemalatha et al., 2007; Jambunathan, 1980; Kayodé et al., 2006; Léder, 2004; Lestienne et al., 2007; Malleshi and Klopfenstein, 1998; Ravindran et al., 1994; Shawrang et al., 2011).

Additionally **Table 1.6** shows an overview of the PC which have been found in sorghum and millets and their potential for mineral binding. An important remark is that these tables show ranges of reported values of minerals and antinutritional factors in sorghum and millets and that vast differences exist between different varieties of these cereal grains. The high differences in minerals and antinutritional factors gives possibilities to screen varieties which are better suited for improved mineral bioavailability.

Plenty of work has been done concerning the microbiota of African fermented porridges but the functionality of the microbiota is still an area requiring further research. **Table 1.7** provides an overview of microorganisms reported in the most common African fermented porridges used for complementary feeding along with the known phytase and PC conversion activity. This information, albeit limited, can be used to produce functional starter cultures for traditional fermented cereal porridges. A more thorough characterization of these microbiota and their functional characteristics is urgently needed and especially how the fermentation process could be optimized at a household level. Many studies have demonstrated the reduction of PA and PC after spontaneous fermentation of African porridges and we will look to a few examples of these products to determine if the reduction is sufficient to cause a significant change in mineral bioavailability.

Table 1.6: Types and levels of phenolic compounds in sorghum and millets

Phenolic compound	Ability to bind minerals	Sorghum	Millets
Gallic acid	Yes	ND-130.6	1.8-6.2
Protocatechuic	Yes	7.4-141	1.6-120
p-Hydroxybenzoic acid	No	4-24.2	6.3-126
Vanillic acid	No	ND-50	15.2-168.6
Caffeic acid	Yes	3.4-52	3.9-339
p-Coumaric acid	No	6.4-200	36.9-2133.7
Ferulic acid	No	8.9-500	285-2209
Cinnamic acid	No	4.7-19.7	35.1-781 -7
Gentisic acid	Yes	-	8.3-168.6
Syringic acid	No	38.8	6.2-141.4
Chlorogenic acid	Yes	-	3.6-19
Sinapic acid	No	50-140	0.8-55
Luteolinidin	Yes	ND-283	-
Apegenidin	Yes	ND-1570	-
5-Methoxyluteolidin	Yes	ND-153	-
7-Methoxyapigenidin	Yes	ND-137	-
Eriodictyol	Yes	ND-997	-
Naringenin	Yes	ND-639	20.93-85.81
3-Deoxyanthocyanin	Yes	ND-187	-
Luteolin	Yes	3-75	-
Apeginin	Yes	ND-288	15.53-101.96
Quercetin	Yes	22.1	-
Proanthocyanidins	Yes	ND-68000	-

Values are expressed in µg/g. Retrieved from: (Dicko et al., 2006; Dykes et al., 2011; Dykes and Rooney, 2006; Dykes et al., 2013; Dykes et al., 2009; Pradeep and Sreerama, 2015; Shahidi and Chandrasekara, 2013; Shen et al., 2013)

Table 1.7: Microbial communities of some African cereal fermented gruels used for complementary feeding

Fermented Food Product	LAB and yeasts	Microorganisms with phytase activity	Microorganisms with PC conversion activity
Degué	*Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus gasseri, Lactobacillus plantarum, Enterococcus* sp.	-	-
Ben-saalga	*Lactobacillus fermentum, Lactobacillus paraplantarum, Lactobacillus plantarum, Lactobacillus salivarius, Pediococcus acidilactici, Pediococcus pentosaceus*	*Lactobacillus plantarum* 4.4 and 6.1, *Lactobacillus fermentum* 7.4	-
Gowé	*Lactobacillus fermentum, Weissella confusa, Lactobacillus mucosae, Pediococcus acidilactici, Pediococcus pentosaceus, Weissella kimchii Kluyveromyces marxianus, Pichia anomala, Candida krusei, Candida tropicalis*	-	-
Koko	*Lactobacillus fermentum, Lactobacillus paraplantarum, Lactobacillus salivarius, Pediococcus* sp., *Weissella confusa*	-	-
Togwa	*Lactobacillus brevis, Lactobacillus cellobiosus, Lactobacillus fermentum, Lactobacillus plantarum, Pediococcus pentosaceus Candida glabrata, Hanseniaspora guilliermondii, Issatchenkia orientalis, Pichia anomala, Pichia burtonii, Pichia*	*Hanseniaspora guilliermondii, Issatchenkia orientalis, Kluyveromyces marxianus TY04TY14 and TY20, Pichia*	-

	guilliermondii, Pichia kudriavezvii, Pichia norvegensis, Kluyveromyces marxianus, Saccharomyces cerevisiae	*anomala TY06, Pichia kudriavzevii TY13*	
Ting	*Enterococcus faecalis, Enterococcus mundtii, Lactobacillus casei, Lactobacillus coryniformis, Lactobacillus curvatus, Lactobacillus harbinensis, Lactobacillus fermentum, Lactobacillus parabuchneri, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactococcus lactis, Weissella cibaria*	-	*Lactobacillus casei, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus reuteri*
Obushera	*Enterococcus* sp.*, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus reuteri, Lactococcus lactis, Leuconostoc lactis, Pediococcus pentosaceus, Streptococcus gallolyticus, Streptococcus infantarius, Weissella confusa*	-	-
	Clavispora lusitaniae, Cyberlindnera fabianii, Issatchenkia. orientalis, Saccharomyces cerevisiae		
Kunu-zaki	*Lactobacillus delbrueckii, Lactobacillus fermentum, Streptococcus bovis, Streptococcus gallolyticus, Streptococcus lutetiensis, Weissella confusa*	-	-
Ogi	*Lactobacillus amylolyticus, Lactobacillus bifermentans, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus helveticus, Lactobacillus nantensis, Lactobacillus*	*Saccharomyces cerevisae, Candida krusei, Candida tropikalis, Geotricum candidum*	-

	pantheris, Lactobacillus plantarum, Lactobacillus vaccinostercus, Weissella confusa		
	Candida krusei, Candida tropicalis, Geotricum fermentans, Geotricum candidum, Rhodotorula graminis, Saccharomyces cerevisiae		
Munkoyo/chibwantu	*Acetobacter lovaniensis, Lactobacillus casei, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus helveticus, Lactobacillus rossiae, Leuconostoc pseudomesenteroids, Streptococcus gallolyticus, Weissella cibaria, Weissella confusa*	-	-

Retrieved from: (Abriouel et al., 2006; Greppi et al., 2015; Hellström et al., 2015; Hellström et al., 2010; Lei and Jakobsen, 2004; Madoroba et al., 2011; Mugula et al., 2003; Mukisa et al., 2012; Oguntoyinbo and Narbad, 2012; Oguntoyinbo et al., 2011; Omemu et al., 2007; Schousta et al., 2013; Sekwati-Monang and Gänzle, 2011; Songre-Ouattara et al., 2008; Turpin et al., 2011; Vieira-Dalodé et al., 2007).

1.6.1 Reduction of iron and zinc binders in naturally fermented African cereal porridges

The reduction of mineral binders during natural fermentation seems to be dependent on a variety of factors. The reduction of PA in African fermented foods is highly varied with some authors finding no degradation (Onyango et al., 2005; Songre-Ouattara et al., 2010), less than 50% degradation (Antony and Chandra, 1998; Kruger et al., 2012; Proulx and Reddy, 2007; Towo et al., 2006) and others finding more than 50% degradation (Kayodé et al., 2013; Kruger et al., 2012; Mouquet-Rivier et al., 2008; Proietti et al., 2013; Tou et al., 2007a; Towo et al., 2006). Several factors seem to influence the reduction of mineral binders in African fermented foods. The first one is related to processing conditions. In general, processing conditions that include soaking, germination (or addition of small amounts of malt) and methods that generate waste streams such as dehulling and sieving result in the highest reduction of mineral binders. Most studies have shown that germination can increase iron and zinc availability by causing an increase in the endogenous phytase activity owing to *de novo* synthesis and/or activation of the endogenous phytases (Agostini et al., 2010; Gibson et al., 1997; Gibson et al., 2006; Traoré et al., 2004). Milling and sieving also reduces the PA content since a large proportion of PA is present in the bran, which is often sieved off. However, it should be mentioned that these processes also cause simultaneous reduction in both PA and minerals. Changes occurring to PC and dietary fibers after processing conditions such as soaking and germination in relation to mineral bioavailability are not much documented.

The second factor is related to fermentation conditions. Longer fermentation time favors more degradation of PA. After 72 h of fermentation 68% reduction in PA was observed vs. 22% reduction after 14 h of fermentation during the production of *gowé* (Kayodé et al., 2013). Other authors have also concluded that when longer germination time and fermentation time are combined, more than 50% reduction of PA is observed in sorghum and finger millet porridges (Kayodé et al., 2013; Kayodé et al., 2006; Makokha et al., 2002). The last factor is dependent on the matrix being used for the fermentation and also the variety of grains used. Natural fermentation of *bensaalga* showed reduction of PA but use of backslopping inoculum from *bensaalga* to ferment a pearl–millet soybean slurry failed to show any PA reduction. The authors attributed this phenomenon to the presence of one or more antinutrients in the soybean. During the fermentation of sorghum, it was observed that there was more reduction of PA (68-72%) in the non-tannin sorghum than in the tannin sorghum (17-24%) (Kruger et al., 2012). Differences in PA reduction were also observed during co-fermentation of pearl-millet with groundnut (Tou et al., 2007a). These differences clearly highlight that some matrices favor the reduction of PA than others. Proietti et al. (2013) found PA degradation between 58–72% of several fermented porridges produced from different sorghum varieties. The endogenous phytase activity of

the sorghum flours varied between 0.17–0.21 PU/g and increasing to 0.64–3.44 PU/g in the fermented slurries and this demonstrates that PA reduction is also variety dependent. Baye et al. (2013) also found that *injera* made from wheat and/or barley as one of the flour blends showed a complete hydrolysis of phytate whereas only 28 % hydrolysis was found for teff-white sorghum *injera*. Sorghum and millets thus pose challenges of low phytase activity, high PC especially CT which should be dealt with in order to sufficiently increase the mineral bioavailability.

The reduction of CT and PC has also been observed after fermentation. What is consistent among all workers is that a reduction in tannins alone without sufficient reduction in PA does not significantly improve the solubility and bioavailability of iron. Also, there is always a higher mineral bioavailability in the non-tannin porridge compared to the tannin counterpart (Antony and Chandra, 1999; Kruger et al., 2012; Towo et al., 2006). No significant improvement is observed in mineral bioavailability in tannin porridges sometimes even after a reduction of the tannins probably because the tannins may be able to bind with other food components such as proteins forming insoluble complexes thus making them less assayable (Matuschek et al., 2001). Reduction in dietary fibers has also been observed during the preparation of *ben-saalga* (Mouquet-Rivier et al., 2008; Tou et al., 2007a). The effect however, on the mineral bioavailability is not clear.

Another important factor to consider during the production process of African fermented porridges is the cooking process. Cooking has shown inconsistent results i.e. no change in the level of PA (Kayodé et al., 2007b; Kruger et al., 2012) and PC (Kayodé et al., 2007b) or slight decreases in PA content after cooking fermented sorghum and millet flours (Kruger et al., 2012). The changes can be attributed to differences in cooking conditions and the (re)use of the cooking water. Most of these processes seem to have an impact on the PA level but there are inconsistencies regarding to the changes in total PC possibly due to differences in the plant matrices. Recent studies have shown that reduction of PA alone in unrefined cereals normally used in Africa, is not sufficient to increase mineral bioavailability and it is suggested that there could be an interaction of PA, PC and dietary fibers. To test this hypothesis, Baye and coworkers (2015) used exogenous phytase to degrade PA, polyphenol oxidase to degrade PC, and xylanase and cellulase to degrade dietary fibers in *injera* made from different flour blends. Their findings indicated that iron bioaccessibility was higher in the flour blend where a combination of all the enzymes were used. They also showed that reduction of dietary fibers increased iron bioaccessibility independent of PA reduction. The role of dietary fibers in mineral absorption thus deserves further attention.

1.6.2 Reduction of mineral binders in relation to mineral bioavailability in African cereal fermented porridges

While it is quite clear that fermentation has great potential in reducing mineral binders, perhaps the question that still remains to be answered is whether this reduction is sufficient to cause significant improvement in mineral bioavailability. Many studies already mentioned above, have seen an increase in iron and zinc solubility and dialyzability after fermentation with the assumption that an increase in mineral solubility causes increase in bioavailability. Hurrell and coworkers (2010) suggested that the molar ratio of PA/Fe should be < 1 or optimally < 0.4 for meals that are based on plain cereals and/or legumes with no enhancers of iron absorption such as ascorbic acid and meat.

While many studies on African fermented porridges have seen increases in solubility of iron and zinc after fermentation, these results have to be interpreted carefully as vast differences exist between *in vitro* and *in vivo* studies. For example, a human study of 78 subjects carried out by Hurrell et al. (2003) showed that complete dephytinization of low tannin non-fermented sorghum porridges, by the addition of exogenous phytases, increased iron absorption by 2 fold. However, in a high tannin sorghum variety only 21-34% increase in iron absorption was observed. Despite the porridges having a PA/Fe ratio of close to 0, no significant improvement in iron absorption was observed in the high tannin sorghum. While several authors have found several folds increases of *in vitro* soluble iron after incomplete dephytinization by fermentation (Kayodé et al., 2006; Kruger et al., 2012; Towo et al., 2006) this may possibly mean not more than 1% bioavailability if we compare with *in vivo* studies and this is certainly not adequate for improved iron nutrition. Hurrell et al. (2003) suggested that degradation of more than 90% PA is required in order to have a 2-fold increase in iron bioavailability. However, it still remains a question if we can compare the results of *in vivo* studies done on non-fermented products with fermented products. According to Proulx and Reddy (2007), an acidic environment may be more important for iron solubility and absorption as it has an enhancing effect on the ferric iron transport across the intestinal wall.

The high tannin sorghum variety used in the *in vivo* study of Hurrell et al. (2003) had 3.36% CT while the low tannin varieties contained < 0.039%. It may be suggested that in cereal grains such as sorghum and millets which may contain CT, a maximum amount of 0.03% CT may be necessary for improved iron absorption after dephytinization. Cercamondi et al. (2014b) also found the same effect of different concentrations of added PC on iron absorption in non-fermented sorghum porridges in a human study of 50 participants. They suggested that sorghum porridges with 80-160 mg gallic acid equivalents (GAE) PC have limited iron bioavailability even if most of the PA is degraded. That means that a maximum of 80 mg GAE PC may be needed for improved iron absorption in sorghum porridges.

Pertaining to zinc, *in vitro* studies seem to correlate well with *in vivo* studies. A zinc absorption of 9.1% for maize porridge (PA/Zn = 9.2), 10.7% for white sorghum porridge (PA/Zn = 11.6), 8.4% for brown sorghum porridge (PA/Zn = 9.2) (Brnić et al., 2014), 8.2% for whole wheat (PA/Zn = 8.6) (Sandstrom, 1992) and 13.8% for yeast fermented wheat bread (PA/Zn = 0.5) has been observed. Although PA/Zn is much lower for some cases, it is not accompanied by much higher zinc absorption. The study of Brnić et al. (2014) showed that after dephytinization of meals zinc absorption is less inhibited by PC. A decrease in zinc absorption was observed in high tannin sorghum vs low tannin sorghum. Zinc absorption decreased from 10.7% in dephytinized tannin sorghum to 8.4% in the non-dephytinized counterpart. The authors attributed this phenomenon to the possible interaction of PC with zinc and PA which are absent when PA is degraded. Nonetheless, these results show that the degradation of PA is necessary to improve zinc absorption in humans.

Table 1.8 shows a list of the most common fermented porridges consumed in Africa along with their antinutrient content and PA: mineral ratios. If we are to consider that 0.03% CT, < 80 mg GAE total PC, < 0.4 PA/Fe and < 5 PA/Zn is needed for an improved iron and zinc bioavailability, then the iron and zinc bioavailability of African fermented porridges available at this moment is estimated to be very low. From Table 1.7 it can be observed that most porridges contain high levels of PC and CT thus iron and zinc absorption will likely be not more than 1 and 5% respectively. The absolute absorption of iron from cereal porridges is known to be as low as 1% while that of zinc may be over 5% (Brnić et al., 2014). The absolute absorption of iron may be low because the absorption of iron is dependent on several factors such as level of inhibitors (PA, PC and dietary fibers), host factors (iron status, nutritional deficiencies, genetic disorders, gastric and intestinal pH) while zinc absorption may also be affected by host factors but dietary factors are most likely the primary causes (Hotz and Brown, 2004; Hurrell and Egli, 2010). Based on the values in Table 1.8, we can conclude that none of these porridges have the optimal PA: mineral ratio required for adequate iron and zinc absorption as such more efforts are required to improve mineral bioavailability.

1.6.3 Use of functional starter cultures in African fermented cereal porridges

To achieve sufficient reduction in mineral binders and enable a predictable and controllable fermentation, use of functional starter cultures is probably necessary. We have already shown that more than 90% reduction of PA is needed to have significant improvement of iron and zinc bioavailability but this may be impossible to achieve by natural fermentation. A further reduction is also required for the PC especially the CT. The use of starter cultures has been demonstrated in the successful fermentation of *bensaalga* (fortified with soybean or groundnut) with high energy density (Songré-Ouattara et al., 2009; Songre-Ouattara et al., 2010). The starter culture used for the *bensaalg*a

fortified with soybean also had phytase activity and showed no reduction in PA content even though it had been isolated from a traditionally produced *bensaalga* where PA reduction had occurred (Songre-Ouattara et al., 2010). This suggests that the phytase producing activity of the starter culture is dependent on the food matrix and that further investigation for different common food matrices is required. As mentioned earlier, most of the African fermented porridges have low nutrient content as a result of the low DM as such cofermenting with legumes has been suggested as a way to improve the nutrient density. The most common legumes that can be associated with cereals include soybean, groundnut and cowpeas. It thus means that studies on different matrices is required to get a better understanding of how the microbial ecology is influenced by different food matrices.

To our knowledge, no data on porridges based on maize, sorghum and millets have been published showing more than 90% reduction of PA and reasonable reduction in PC after using a starter culture. The use of starter cultures with functional purpose has been a subject of importance over the past decades but until now, the production of African fermented products still remain highly an artisanal activity with the use of rudimentary conditions. This is most likely because of the challenges presented in an African setting such as high cost of production, stability of cultures in areas where there is no cold storage which influence maintenance and distribution and lack of educative initiatives to make people more aware of improved fermentation technology. The high cost of production associated with the use of starter cultures means that this may only be appropriate for small and medium production units who may be interested in a starter culture that allows for accelerated acidification, improved nutritional value without affecting the price and sensory attributes. But how about populations who rely solely on small scale household production? The application of starter cultures in households in a way that is easily understood, culturally feasible and affordable should be of highest priority.

The use of backslopping is beneficial for products that are consumed every day and produced by the same type of cereals. This is because the use of continuous backslopping can result in the dominance of the best adapted strains and if these strains are of functional interest, then it will be highly relevant (Holzapfel, 2002). For some products such as *ogi, togwa* and *obushera*, where different food matrices and variations can be used, backslopping may present challenges as the microbiota may not be well adapted to all the variations in production. The use of starter cultures has been very effective in Europe in delivering products of high quality and nutritional value but it still remains elusive in Africa. Several hurdles still have to be overcome for this to be effective. Robust field techniques may be necessary to monitor critical steps in the fermentation process of African products (Holzapfel, 2002) as most studies have demonstrated vast differences in laboratory produced products vs. field produced products.

Table 1.8: Iron, zinc, antinutrients and estimates of bioavailability of African sorghum and millet fermented porridges/gruels

Type of porridge	Iron	Zinc	Fiber	PA	PC	CT[1]	PA/Fe	PA/Zn
Ogi/koko	1.31	1.19 - 1.4	-	188	-	-	12.2	14-15.6
*Fermented sorghum porridge (non-tannin variety)	5.4	-	-	212	-	-	3.3	-
*Fermented sorghum porridge (tannin variety)	5.8	-	-	1241	-	330	18.2	-
[2]*Fermented sorghum porridge	3.2-9.9	1.5-2.5	-	3.7-962	62-212[b]	-	0.03-22.46	0.27-55.9
Ben-saalga	8.33	2.05	2140	220	393[b]	-	2.2	10.6
Togwa	3.6-23.8	0.9-1.82	-	363-660	-	-	2.4-8.6	33.9-39.7
Gowé	3.53	-	-	369-1003	233-372	176-430	8.7-23.7	-

PA: phytic acid, PC: phenolic compounds, CT: condensed tannins, [1]mg/100 g dm catechin equivalents. All other values are in mg/100 g dm, [2]range of values based on different sorghum varieties, [b]iron binding phenolic groups, *Porridges prepared at laboratory scale simulating African fermented porridges preparation process.

Retrieved from**:** (Adeyeye et al., 2000; Hellström et al., 2010; Kayodé et al., 2013; Kayodé et al., 2006; Kruger et al., 2012; Mouquet-Rivier et al., 2008; Ndabikunze et al., 2001; Olayiwola and Okhiria, 2012; Proietti et al., 2013; Svanberg et al., 1993; Tou et al., 2007b; Towo et al., 2006; Vieira-Dalodé et al., 2007).

1.7 Bioavailability vs. Bioaccessibility

Bioavailability is defined as the amount of consumed nutrient that is digested and released from the food matrix and subsequently absorbed by intestinal cells and transported to body cells where it is available for physiological functions. As bioavailability has a physiological end point, it is dependent on many factors that cannot be mimicked by *in vitro* methods in particular host factors such as nutrient status, age, genotype etc. (Etcheverry et al., 2012; Rein and da Silva Pinto, 2017). Bioaccessibility on the other hand, is the amount of ingested nutrient that is digested and released from the food matrix and is thus potentially available for absorption (Cardoso et al., 2015; Rein and da Silva Pinto, 2017). Bioaccessibility is only dependent on digestion and release from the food matrix as such it can be

mimicked through the use of *in vitro* methods and can be used to investigate the effect of factors such as food processing, nature of food matrix and effect of luminal factors such as pH. It is thus a useful tool to characterize foods on their approximate bioavailability and also can be used as screening and ranking tool. *In vivo* methods are the only methods which can accurately determine bioavailability but these methods require a lot of expertise, are time consuming, very expensive and present ethical challenges that have to be considered (Cardoso et al., 2015; Etcheverry et al., 2012).

In vitro methods are thus widely used to estimate bioavailability although they can never substitute *in vivo* methods because of the complexity of the digestion process which can never be fully mimicked *in vitro*. Solubility is one method that has been used to determine bioaccessibility of minerals such as iron and zinc. This method is relatively easy to perform and inexpensive as it only requires digestion of the food using gastric and intestinal enzymes at the appropriate pH (Etcheverry et al., 2012). Solubility measurement can be coupled with dialyzability whereby the soluble minerals are allowed to pass through a dialysis membrane with a specific molecular weight cut-off following the gastric digestion. This method was introduced by Miller et al. (1981) and is based on the fact that small molecular weight compounds can be dialyzed and are likely to be available for absorption in the small intestine. Both solubility and dialyzability methods cannot measure the rate of uptake of the mineral and also the effect of competition at the site of absorption so careful interpretation of these results is required. It has been suggested that dialyzability assays for zinc are a good estimator of zinc bioavailability (Etcheverry et al., 2012) while for iron, several findings have indicated a strong correlation between iron bioavailability and dialyzability studies although in some cases the magnitudes were different and also depended on the parameter that was tested (Aragón et al., 2012; Sandberg, 2005; Walter et al., 2003). However, it is generally accepted that the direction of response of iron dialyzability always corresponds to bioavailability studies which proves the usefulness of this method to be used for baseline studies (Bohn et al., 2017). Gastrointestinal models which are dynamic *in vitro* models take into account many parameters such as peristalsis, churning and body temperature but are also very expensive and require validation studies (Etcheverry et al., 2012). The most reliable method that can be used to estimate bioavailability is the coupling of the above mentioned methods with Caco-2 cell model which involves the use of Caco-2 cells which belong to the human epithelial cell line and behave like intestinal cells when cultured (Glahn et al., 1998). Caco-2 cells are able to measure transport and competition at the site of absorption and are widely used to determine the bioavailability of iron which is indicated by the formation of ferritin by the Caco-2 cells (Etcheverry et al., 2012; Glahn et al., 1998). There are not many studies concerning the use of Caco-2 cells to determine the bioavailability of zinc as there are challenges in the identification of appropriate biomarkers for zinc.

Bioaccessibility can be measured by both solubility and dialyzability but these two measures are in fact different and not comparable as solubility is rather a crude measure of bioaccessibility which includes both high and low molecular weight iron or zinc, while dialyzability only includes low molecular weight iron or zinc which is likely to be absorbed. Dialyzability is thus a better measure of bioaccessibility and throughout this PhD, bioaccessibility is defined as the proportion of minerals able to pass through a dialysis membrane of 12-14 molecular weight cut-off. In addition, the term bioaccessibility will only be used to refer to cases where dialyzability was used (or other cases where low molecular weight iron or zinc was measured by other methods such as ultra-filtration) while solubility will be referred to as such. The term bioavailability will be used when Caco-2-cells, human and animal studies were used and also to refer to the general context of the subject matter.

1.8 Probable reactions of iron and zinc during digestion

One of the reasons why bioavailability of iron is a challenge is attributed to the complex redox reactions undergone by iron at different pH and redox potentials. **Figure 1.3** shows the Pourbaix diagram for 1 M iron solutions depicting the fate of iron at different redox potentials and pH. The Pourbaix diagram allows the prediction of the dominant species of iron that will exist at certain levels of redox potentials and pH (Delahay et al., 1950) implying that we can predict the fate of iron during digestion. From **Figure 1.3**, it can be observed that in an aqueous system, Fe^{3+} can only exist in its ionic form without precipitation at narrow pH ranges (less than 2) and very high redox potential (about +0.8 to +2.0 volts). Outside this range, Fe^{3+} is reduced to Fe^{2+} or precipitated by hydroxide and oxide ions. The bioavailable form of iron, (Fe^{2+}), is soluble up to pH of around 6.5 and a redox potential of between -0.4 and +0.8 volts.

During gastric digestion, the pH increases depending on the buffering capacity of the food consumed and in the case of a Western type diet comprising of vegetable purees and cocoa based beverages, pH can increase to above 5 (Guerra et al., 2012; Minekus et al., 2014). Hydrochloric acid is secreted in order to maintain an acidic environment optimal for enzymatic activity and a mean pH value of 3 is suggested during gastric digestion (Minekus et al., 2014). The average redox potential during gastric digestion is +0.2 volts (Sousa et al., 2008). On the Pourbaix diagram in **Figure 1.3**, a depiction is given of the predominant form of iron that will exist at this combination of redox potential and pH (redox potential of +0.2 volts and pH 3) in the stomach. At this state, iron will exist mostly in the soluble Fe^{2+} state. Iron in food exists mainly in the Fe^{3+} form, so during digestion, due to the low pH and reducing power of food components such as ascorbic acid, Fe^{3+} is reduced to Fe^{2+}. Fe^{2+} remains soluble until a pH of around 6.5 (and a redox potential of < +0.2) after which it is precipitated as hydroxides.

A lower pH of less than 6.5 is thus required to keep Fe^{2+} soluble and this phenomenon explains why people with achlorhydria (a disease whereby there is reduced hydrochloric acid secretion) suffer from iron deficiency because gastric digestion occurs at higher pH (pH > 4) (Seo et al., 2015; Sharp and Srai, 2007) whereby a narrow redox potential is needed to keep iron soluble. This means that foods with a buffering capacity that can keep the gastric digestion at a pH close to 2 are likely able to keep more iron in the soluble state. Presence of reducing agents in the food such as ascorbic acid, organic acids, and sulphur containing peptides and amino acids help to reduce Fe^{3+} to Fe^{2+} (Miller and Berner, 1989).

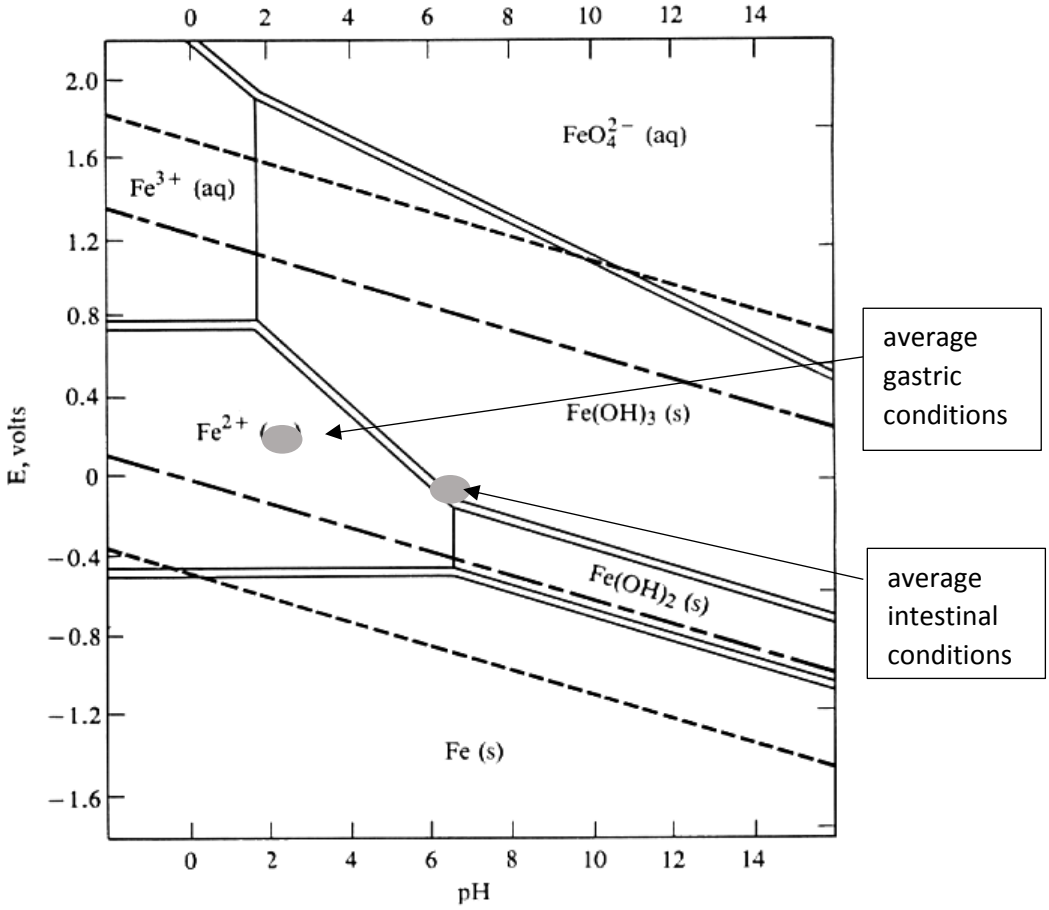


Figure 1.3: Pourbaix diagram for 1 M iron solutions at 25 °C

Retrieved from: Parga et al. (2012)

Solid lines separate species related by acid-base equilibria. Solid double lines separate species related by redox equilibria. Longer dashed lines enclose the theoretical region of stability of water to oxidation or reduction while shorter dashed line enclose the practical region of stability of water. Low E values represent a reducing environment while high E values represent an oxidizing environment. The Pourbaix diagram for zinc is not shown because at both the gastric and intestinal conditions, zinc will be mostly present in its ionic form.

The presence of iron in the soluble state provides an opportunity for iron to be complexed by complexing agents that are released due to the hydrolytic action of hydrochloric acid and pepsin

thereby forming low and high molecular weight complexes of amino acids, peptides, phenolic compounds, soluble forms of PA and other negatively charged molecules (Jacobs and Miles, 1969; Miller and Berner, 1989). The iron released from the food will thus exist as hydrated ions or as soluble complexes (Jacobs and Miles, 1969). The kinetics of release for potential complexing agents from the solid food thereby determine the rate at which Fe^{2+} is bound to soluble complexes. In addition, as gastric digestion proceeds and there is interaction between the solid and the soluble components, there is also likely to be a competition between the complexing agents present in the solid food and those in the soluble fraction. In the case of zinc, since it does not undergo redox reactions, zinc released from the food will be bound in soluble complexes of proteins, PC and soluble forms of PA among other negatively charged molecules. Zinc is likely to be bound in protein complexes since it is mostly associated with proteins (Eagling et al., 2014).

During intestinal digestion, the duodenal pH where mineral absorption takes place is around 6.5-7 depending with the meal type and gastric emptying rate (Ekmekcioglu, 2002; Minekus et al., 2014; Sams et al., 2016) with an average redox potential of -0.066 volts (Sousa et al., 2008). According to the Pourbaix diagram (**Figure 1.3**), at this point, iron will be precipitated as hydroxides. A higher redox potential (up to +0.8 volts) or lower redox potential at this pH of the intestine will still renders iron insoluble. This means that during digestion, complexing agents compete with hydroxide ions and in the absence of complexing agents that can keep iron in the soluble form, iron will be precipitated and thus not available for absorption (Champagne, 1988). In particular, Fe^{3+} has a high affinity for hydroxide ions such that any uncomplexed Fe^{3+} is likely to be precipitated while weak soluble complexes are likely to be replaced by hydroxide ions (Miller and Berner, 1989). At the intestinal pH, the sequestering ability of PA and PC to iron and zinc increases (Champagne and Fisher, 1990; Macakova et al., 2012) such that the corresponding mineral complexes are likely to be absorbed if they are soluble and of low molecular weight. The type and amount of complexing agents in a food thus plays a crucial role in the absorption of iron and zinc. **Figure 1.4** shows the probable reactions that could be taking place during gastric and intestinal digestion.

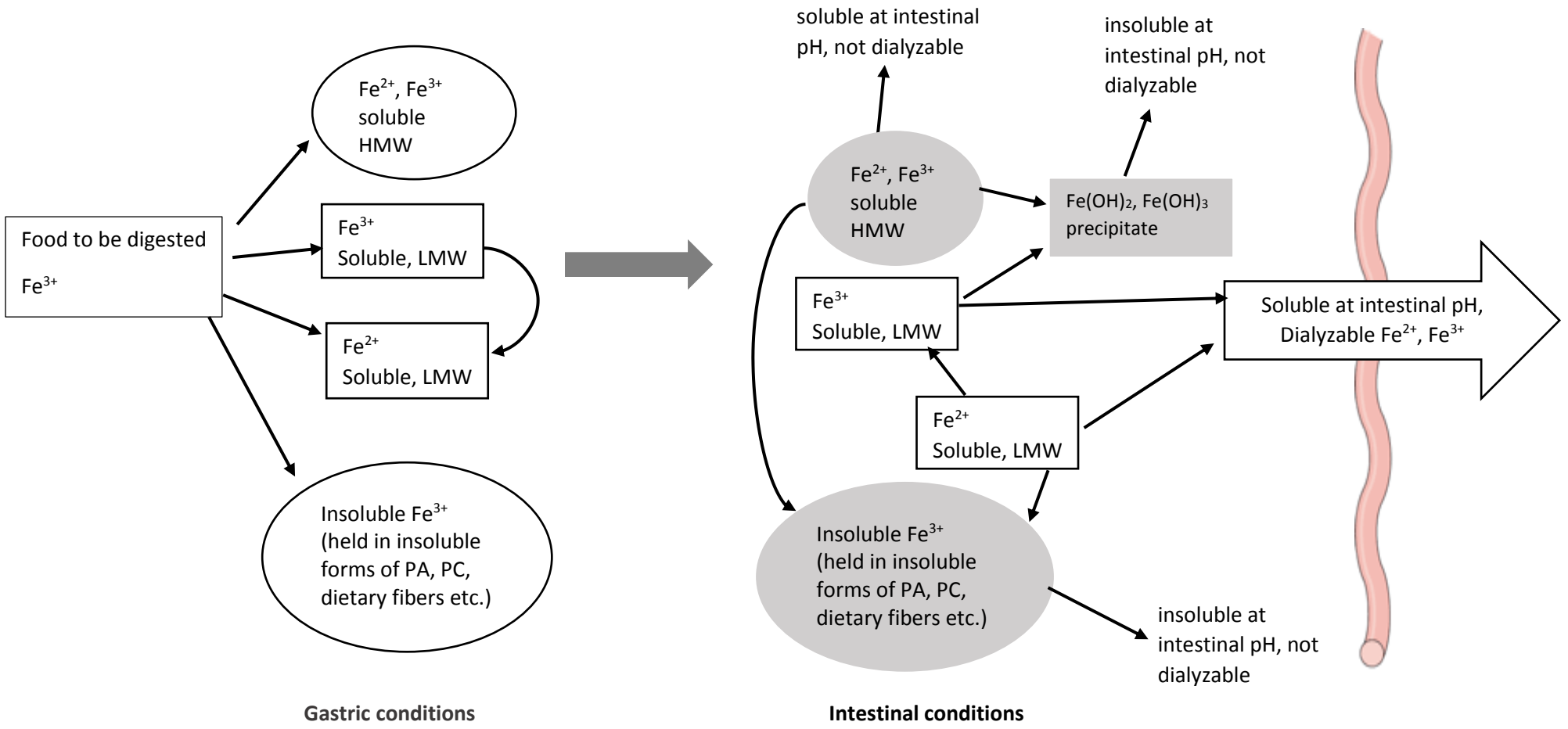


Figure 1.4: Probable reactions of non-haem iron during digestion

HMW: High molecular weight, LMW: low molecular weight

Chapter 2:

An appraisal of the utilization of small seeded grains in Ushe communal area, Zimbabwe

Chapter 2 : An appraisal of the utilization of small seeded grains in Ushe communal area, Zimbabwe.

2.1 Abstract

Small seeded grains such as sorghum, finger millet and pearl millet are important for dietary diversification of communities such as Ushe communal area, in Hwedza district of Zimbabwe whereby maize is the dominant cereal crop. Small seeded grains are also particularly pivotal for the resilience of communities to climate change as these grains are drought resistant. An appraisal of the utilization of small seeded grains through qualitative and quantitative techniques revealed that finger millet was the preferred choice of small seeded grain in this area, used mostly in the preparation of complementary porridges. In addition to the complementary porridges, it was also used in the preparation of thick porridge, non-alcoholic and alcoholic beverages. The processes used in the preparation of the products included germination, fermentation and cooking. Despite the popularity of finger millet for the preparation of complementary porridges, its production was at least six times lower than that for maize and participants attributed this discrepancy to the numerous difficulties encountered during harvesting and processing of finger millet. Moreover, there was lack of information pertaining to the nutritional value of small seeded grains and their usage in other types of products such as gluten free baked products. The need for government intervention is a necessity for policy driven solutions.

Keywords: small seeded grains, finger millet, pearl millet, sorghum, complementary porridge

2.2 Introduction

Sorghum and millets are indigenous cereal grains that have been cultivated and consumed in Zimbabwe and many other African countries since many centuries ago. However, their consumption rapidly decreased in the 1920's after the introduction of maize. The adoption of maize increased rapidly as maize gave higher yields than sorghum and millets and had much lower demands for harvesting and processing (Rurinda et al., 2014a). Sorghum and millets are normally grown in dry regions because of their drought tolerance, but even in some of these areas in Zimbabwe, farmers insist on growing maize creating what is now known as the "maize poverty trap" (Mapfumo, 2009).

Malnutrition especially micronutrient deficiencies are rampant in developing countries. In addition, climate change has rendered many households food insecure. As cereal grains are important staple crops to Africa providing more than 50% of total caloric intake and more than 50% of iron and zinc intake (Joy et al., 2014), the current status quo concerning their cultivation and utilization deserves to be questioned and challenged. The incorporation of sorghum and millets in diets presents many advantages that can help in overcoming the adverse effects of climate change and can contribute to the reduction of malnutrition in developing countries. Sorghum and millets are nutritionally superior to maize as they contain higher mineral contents than maize e.g. finger millet contains ten times more calcium than other cereal grains (Léder, 2004). They also have higher levels of phenolic compounds, many of which have been identified to have therapeutic benefits (Taylor et al., 2014; Taylor et al., 2006).

Ushe is a communal area in Hwedza district of Zimbabwe (18°37'S 31°34'E) characterized with low average rainfall of 750 mm and granitic sandy soils with low organic carbon and low content of nutrients (Rurinda et al., 2014a, b). In Ushe communal area, maize is the dominant cereal crop occupying >80% of the total area under cultivation and is grown for both consumption and/or commercial purposes (Rurinda et al., 2014a). Hwedza district has been selected by the Soil Fertility Consortium of Southern Africa as one of the area which is more vulnerable to climate change as it has recorded the highest increase in temperatures in previous years and also experienced more frequent drought spells than other areas in Zimbabwe (Rurinda et al., 2014b). In addition, Hwedza district is located in a region which recorded the highest stunting in children and highest prevalence of iron deficiency in Zimbabwe (Central Statistics Office, 2011). As consumption of maize alone is clearly not enough to curb the problems of micronutrient deficiencies, this study therefore sought to explore the knowledge and attitudes towards the utilization of small seeded grains in Ushe communal area and to gain an understanding of the cereal grains commonly used in the preparation of complementary

porridges as these are targets for improvement in nutrition programs. Traditional knowledge gained in this study may help in the innovation of products which are culturally acceptable.

2.3 Research Approach

This study was coordinated by SOFESCA who are working in Sub Saharan Africa with particular focus on Hwedza district, Zimbabwe, in an effort to strengthen small holder farming communities resilience to climate change (Mapfumo et al., 2013). Ushe communal area was chosen as the study site for reasons mentioned earlier. Ushe Communal area is located in agro ecological zone 3 (classified mainly according to amount of rainfall) of Zimbabwe (**Figure 2.1**). A combination of qualitative and quantitative data collection tools were used in the form of key informant interviews, learning meetings and one on one interviews in June, 2014. One of the strategies implemented by SOFESCA is the use of learning centers as rallying points for knowledge sharing among community members in order to capacitate communities and reduce vulnerability to the effects of climate change. Key informant interviews were provided by three agricultural extension workers, i.e. people who advise farmers on matters relating to agriculture and live amongst the farmers and one chief (local leader). Ushe communal area consists of 12 villages, with two main market centers. Two learning meetings were conducted at Makurumure shopping center and at St Barnabas shopping center. At least 3 households from each village were present at each learning meeting. However, two villages which were 10 km away from the shopping center only had one household present. An average of 30 households attended each of the learning meetings. About 85% of the attendees were middle aged women as they are more involved in agricultural activities. From the 15% of men in attendance were also village heads i.e. headman, chiefs and councilors. Message to attend the event was disseminated by key informants a week before thus participation was purely by choice and availability of the attendees. Issues discussed fell into four broad categories:

- Type and quantities of small seeded grains grown;
- Type of products normally prepared from small seeded grains and processing methods used;
- Challenges faced during the harvesting and processing of small seeded grains;
- Knowledge about the nutritional information of small seeded grains.

In order to get more specific details about the issues above, one on one interviews were done with 38 households (an average of 3 households per village). The participants were nominated by their peers as people with thorough knowledge of traditional preparation processes of finger millet and were also selected based on two criteria i.e. ease of access (not more than 10 km from main road) and willingness

to participate. The data from the interview was entered and analyzed in SPSS statistics version 22 and are presented in frequency graphs.

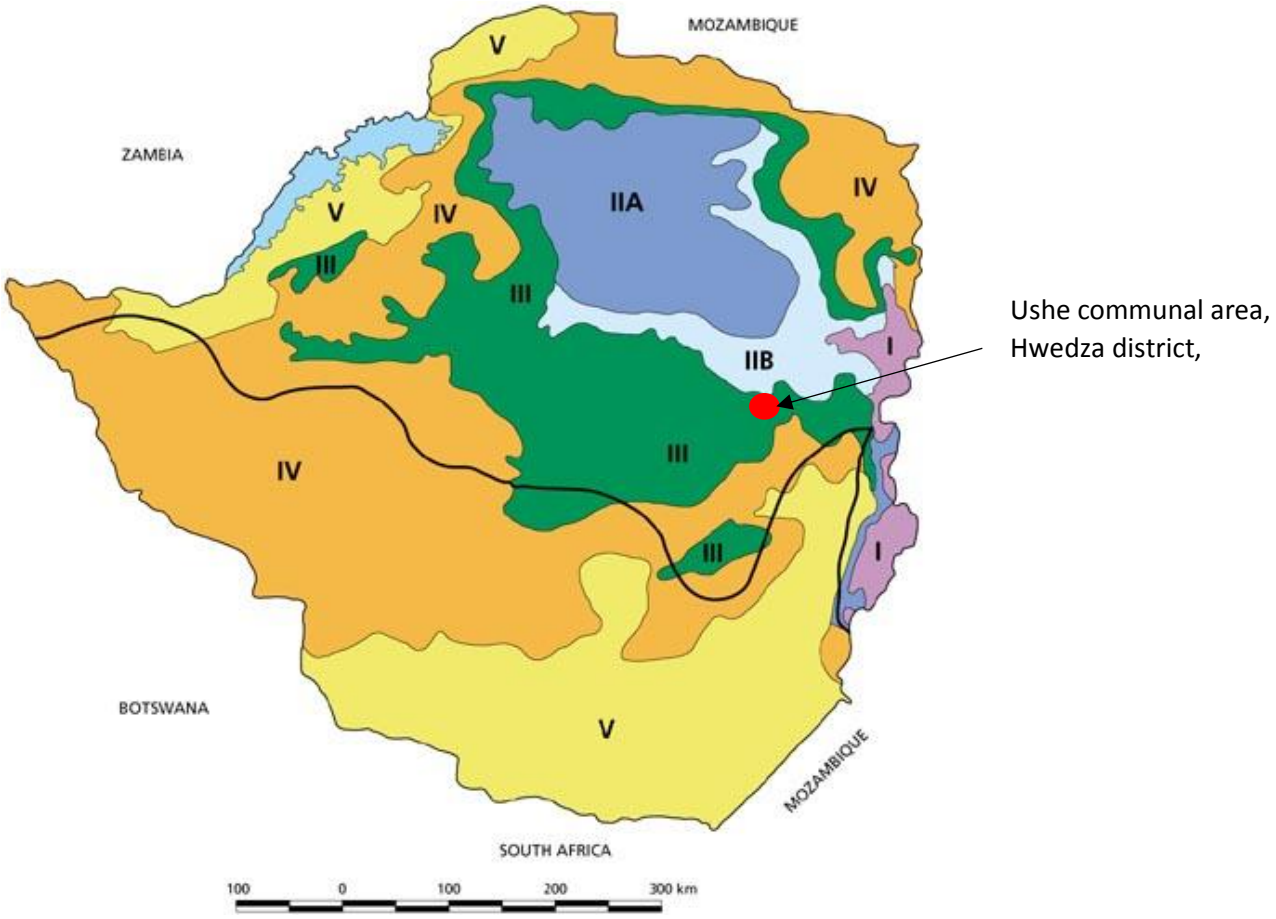


Figure 2.1: Map showing agro-ecological zones of Zimbabwe and the study area, Ushe communal area in Hwedza district of Zimbabwe

Retrieved from: FAO (Corporate Document Repository).

I: Specialized and diversified farming region (rainfall > 1000mm), IIA: Intensive farming region (rainfall 750-1000 mm), IIB: Intensive farming region (rainfall 750-1000 mm), III: Semi-intensive farming region (rainfall-650-800 mm), IV: Semi-extensive farming region (rainfall-450-650 mm), V: Extensive farming region (rainfall <650 mm).

2.4 Results and Discussion

2.4.1 Type and quantities of small seeded grains grown in Ushe Communal Area

The participants for the learning meetings and the interviews were generally women aged between 20-79 with the majority (55%) belonging to the age group 30-59. Less than 5% of the participants were below the age of 30. With the increase of urbanization, more young adults prefer to migrate to the urban areas where they can find employment, as such participation from this age group was low. All the participants were subsistence farmers with farm sizes ranging from 2-5 hectares per household. All the participants had also attained some sort of education (at least primary education) but none had gone through tertiary education.

All the participants grew maize as the major staple and 11% of the participants did not grow any small seeded grains **Figure 2.2).** Of the households that grew small seeded grains, finger millet was the mostly cultivated (94%) followed by sorghum (3%) and pearl millet (3%). From the discussions with key informants and from learning meetings, it was revealed that finger millet was the preferred small grain because it is not attacked by birds as much as sorghum and pearl millet. The majority of the finger millet farmers (80%) cultivated the red finger millet variety which contains condensed tannins (CT), although the white variety is also fairly common. CT offer some protection against bird predation as they are bitter (Dykes and Rooney, 2006). Although red sorghum can also be cultivated because it also contains high level of CT, the preferred sorghum for food preparation in this area was the white sorghum variety. A small number of farmers grew sorghum and pearl millet as it is associated with much higher labor particularly guarding against bird attack during the farming season.

Although, it appears like finger millet is highly adopted by the farmers, the quantities of finger millet harvested differ remarkably from those of maize. **Figure 2.3** shows the distribution of finger millet and maize harvested by all participants. Average amount of maize harvested was 1122 (250-3500) kg/household vs. 164 (20-750) kg/household for finger millet and this was expected as cropping area attributed to small seeded grains ranges from 0-20% (Rurinda et al., 2014a). Amount of maize harvested was almost ten times higher than amount of harvested finger millet. The low production of finger millet can be attributed to both low productivity and cropping area. For example, farmers do not use fertilizers in the cultivation of finger millet compared to maize hence productivity of finger millet is likely to be reduced. In addition, finger millet seeds used are based from the previous harvest and are not treated seeds suggesting a lower yield, whilst for maize, farmers always buy new seed each farming season. In terms of cropping area, a small field is allocated for the cultivation of finger millet and in many cases, the farmers do not take cultivation of finger millet as a priority. The low production

of the small seeded grains means that despite these crops being diversification crops, it may be impossible for them to be included in the diet frequently for a lot of households.

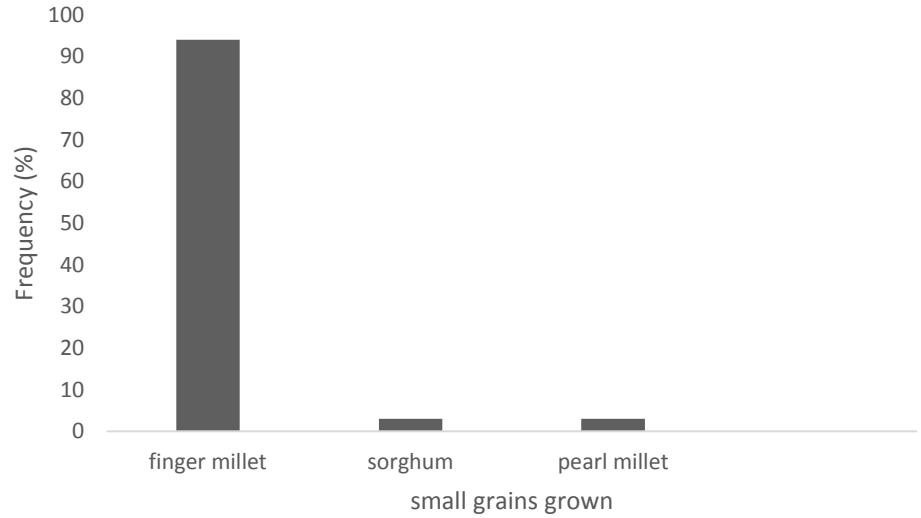


Figure 2.2: Percentage of households cultivating different types of small seeded grains in Ushe communal area, n=38

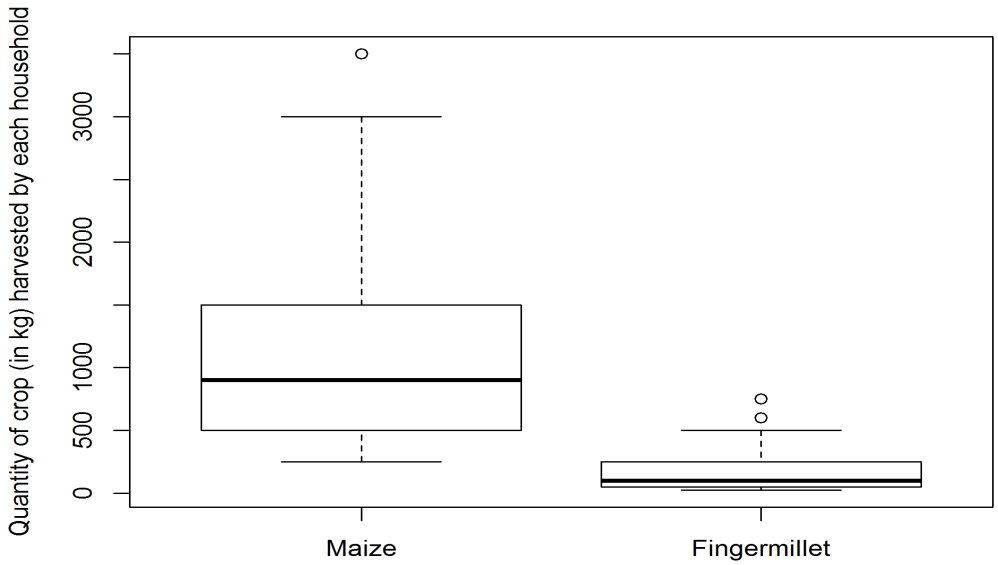


Figure 2.3: Comparison of harvested maize vs. finger millet in the period 2013/14 by the 38 households in Ushe communal area

2.4.2 Type of products prepared from small grains

Four different types of products were generally prepared from finger millet and other small seeded grains. These products included thick porridge or *sadza* which is mainly served in the evening or sometimes in the afternoon (*sadza* is also the main staple prepared mainly with maize), thin porridge or *bota* served in the morning and eaten by both adults and children but most importantly as a complementary food to children and also fed to immunocompromised people. Other products include also non-alcoholic and alcoholic beverages. The non-alcoholic beverage or *maheyu* is a very common fermented food product enjoyed by both adults and children while the alcoholic drink is mainly prepared during traditional ceremonies. **Figures 2.4 and 2.5** show the products that were prepared from small grains and the frequency of consumption of the three main products.

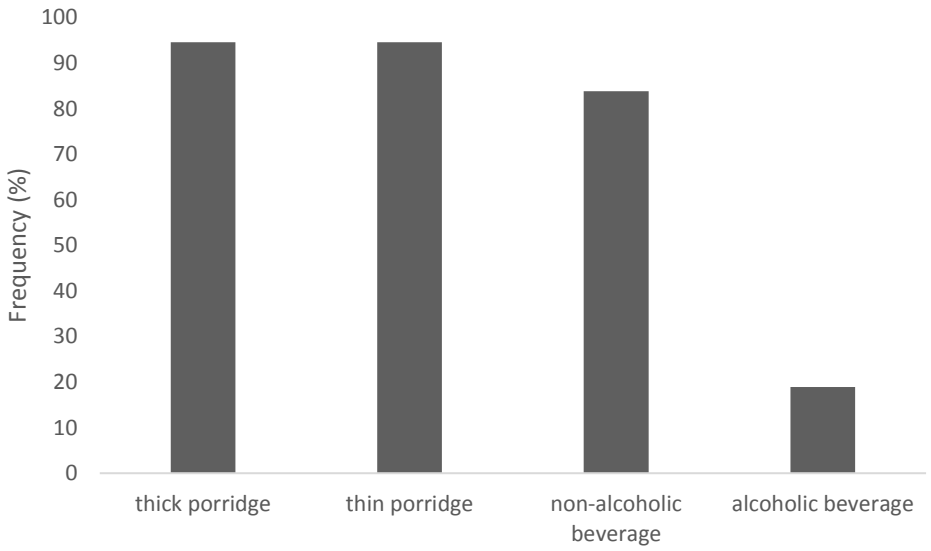


Figure 2.4: Percentage of the type of products prepared from small seeded grains by the 38 households in Ushe communal area

Although the thin and the thick porridge are common products prepared from sorghum and millets, their frequency of consumption is remarkably different. The thin porridge is consumed almost everyday by more than 50% of the participants whilst the thick porridge is consumed occassionally. According to the participants, consumption of the thick porridge is low as most people prefer to serve this dish when there is an animal based relish, in particular chicken. As such the thick porridge is mostly served on special occassions and during traditional ceremonies. However, other participants, particularly those belonging to the older age group, consume the thick porridge almost every week.

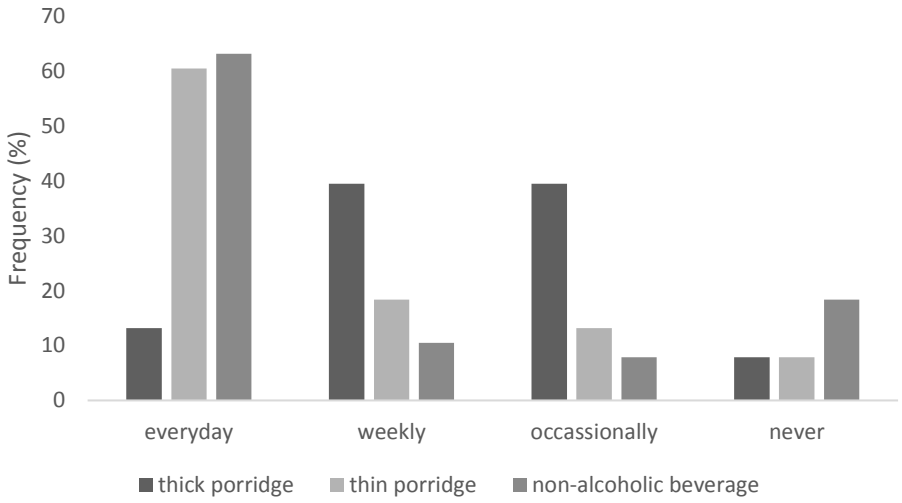


Figure 2.5: Frequency of consumption of thick and thin porridge prepared from small seeded grains by the 38 households in Ushe communal area

Complementary porridges are important as they provide the bulk of the energy and nutrient requirements for the children (Gibson et al., 1998). **Figure 2.6** shows the type of cereal grains that are mainly used in the preparation of complementary porridges. Maize and finger millet are the mostly utilized cereal grains in this area. Finger millet, as it is the mostly grown small seeded grain in this area, is highly used for the preparation of thin porridge. The thin porridge based on finger millet is common as it is believed to be highly nutritious. Although the amount of finger millet harvested is low, it is used almost daily in the preparation of thin porridge. This is likely because only a small amount of the grain is required. For instance, to make the thin porridge, one only requires about 7-10 g of finger millet per 100 g porridge while to make the non-alcoholic beverage, one requires 3-5 g of finger millet malt per 100 mL of the non-alcoholic beverage. The finger millet used in the production of the above mentioned products is generally grown by the participants themselves while those who do not grow the grains basically buy from others. In addition, participants revealed that they can also exchange different crops with each other as a form of batter trade.

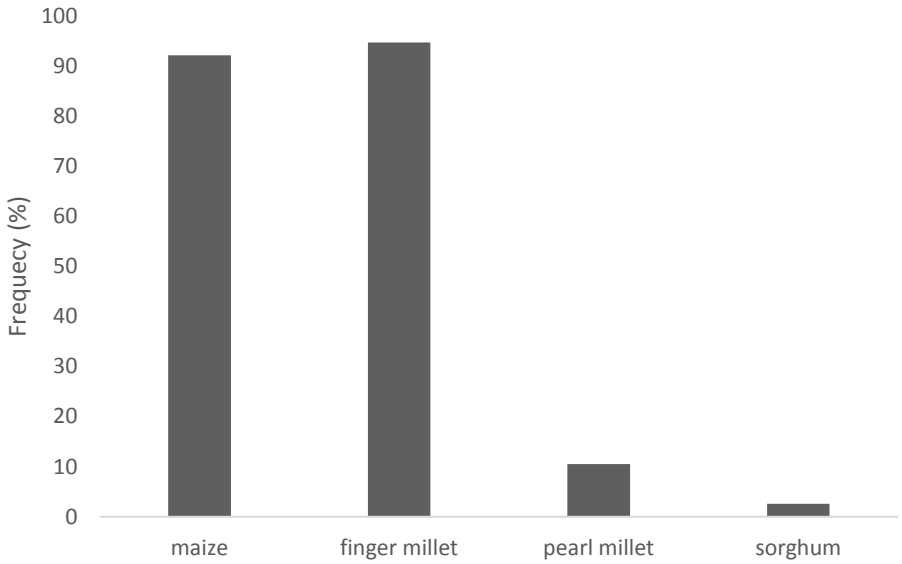


Figure 2.6: Percentage of the types of cereal grains used to make complementary porridges (i.e. thin porridges) by the 38 households in Ushe communal area

2.4.3 Challenges faced in the production of small seeded grains

If the cultivation and consumption of small seeded grains is to be improved, it means that several hurdles encountered during their cultivation and processing should be overcome. According to the participants, there are many challenges faced in the production of small seeded grains. **Figure 2.7** shows some of the challenges faced by the farmers during the whole production of sorghum and millets. The most universal challenge that farmers face is the high labor involved during cultivation, harvesting and processing. Other challenges faced include low yield, lack of mechanization for most of the processing steps as compared to maize and poor acceptability of products by family members. The yield of finger millet has been found to be low in Ushe communal area under different scenarios e.g. different fertilization rates, different planting dates and variable soil nutrient inputs (Rurinda et al., 2014a). Lack of mechanization in the processing of finger millet is of high concern as it not only increases the labor involved in the processing but also affects the quality of the end product. The most critical stage during the processing of the small seeded grains in particular finger millet, is the threshing process whereby the grains are separated from the inflorescence. This is followed by pounding of the grains using pestle and mortar, and winnowing of the grains to remove all foreign particles and glumes. The grains are normally ground at the commercial mill to make flour but some people prefer to make the flour themselves using a traditional buhr mill.

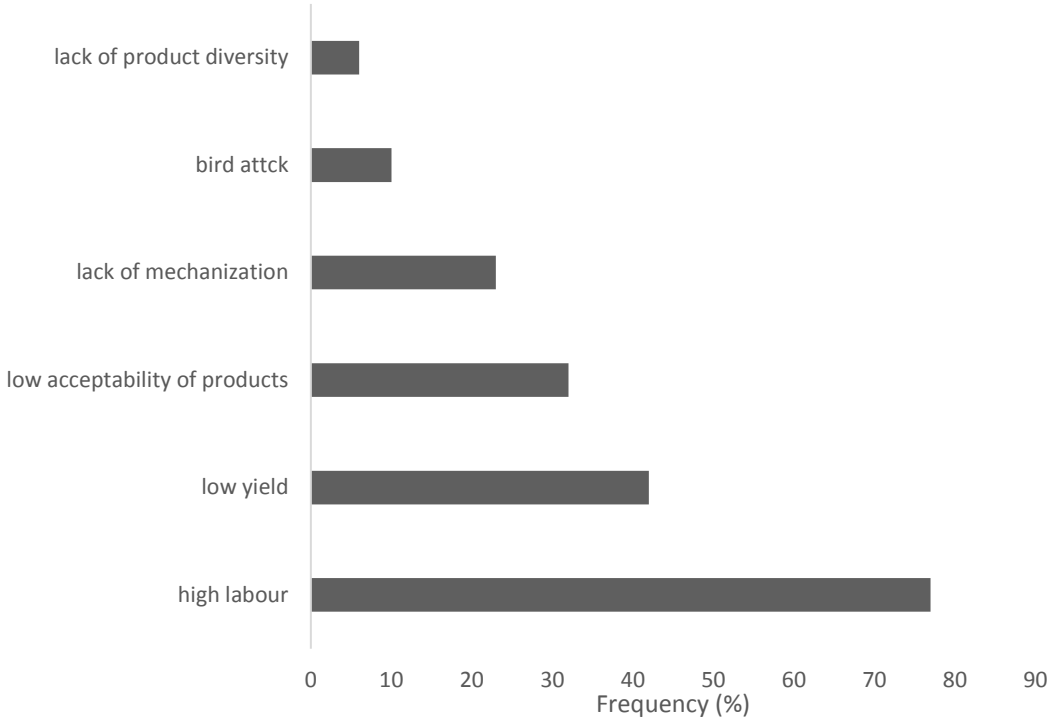


Figure 2.7: Challenges faced during the production of small seeded grains as indicated by the 38 households in Ushe communal area

The products made from small seeded grains are lowly appreciated by the young people as they consider products made from maize, to be better than colored products. For example red colored products from red finger millet and red sorghum and greenish products from pearl millet are considered undesirable. However, participants belonging to the older age group (50+ years) generally do not believe that there are challenges incurred during the cultivation of small seeded grains but that cultivation of these type of cereal grains requires patience and experience.

2.4.4 Knowledge about the nutritional information of small seeded grains

Positive decisions concerning agricultural and consumption practices are likely to be made from a knowledge based perspective. The majority of the participants (97%) believed that small seeded grains are important to achieve more food secure households. This is because small seeded grains specifically finger millet can be stored for up to 5 years without treatment suggesting that finger millet can be used as a backup for drought times which are now recurring predictably in this area. Nevertheless, the challenges in their processing are major deterrents that reduce their cultivation. Only 47% of the participants appreciated the nutritional value contributed by small seeded grains to a diet. The nutritional knowledge about the small seeded grains was based on traditional knowledge whereby it is believed that small seeded grains such as finger millet can be used to cure certain ailments. Indeed

phenolic compounds in finger millet have been identified to have certain therapeutic purposes such anti-oxidative, anti-inflammatory, anti-diabetic and anti-microbial activities (Chandrasekara and Shahidi, 2012; Dykes and Rooney, 2006; Taylor et al., 2014). It also emerged that participants were not aware of the multiple uses of small seeded grains particularly in the production of baked products such as gluten free breads or wheat based products fortified with the small seeded grains (Taylor et al., 2006). The advantage of incorporating small seeded grains in diets in order to complement nutrients that are lacking in maize was also a factor that participants were not aware of. In terms of processing methods used, the two most common processes used were fermentation and cooking during the preparation of thin porridge while germination and fermentation are used in the preparation of the non-alcoholic beverage. The finger millet is used as a whole grain whilst for maize, about 50% of the participants decorticated the grains before milling. Knowledge about the effect of processing on nutritional value of foods was not apparent although a few individuals believed that fermented foods are good for the stomach.

The gap in knowledge aided the formation of four learning centers that were formed out of 24/38 of the women who participated in the interviews. The learning centers are important as it has been found that farmer involvement is important for the acceptance and eventual adoption of innovative ideas pertaining to agricultural and food processing practices (Mapfumo et al., 2013). In addition, innovative ideas may be inspired by the traditional knowledge that exists among the farmers. In line with the above analyzed aspects affecting the utilization of small seeded grains in Ushe communal area, a schematic overview is shown in **Figure 2.8** to explain the challenges that are currently being faced in this area and in many of Sub Saharan Africa as a whole and the possible solutions to these problems. Government intervention is critical to achieve food secure communities through the provision of policies that enable a conducive environment for the cultivation of these small seeded grains such as allowing for fair trade of small seeded grains and providing subsidies that encourage cultivation of small grains.

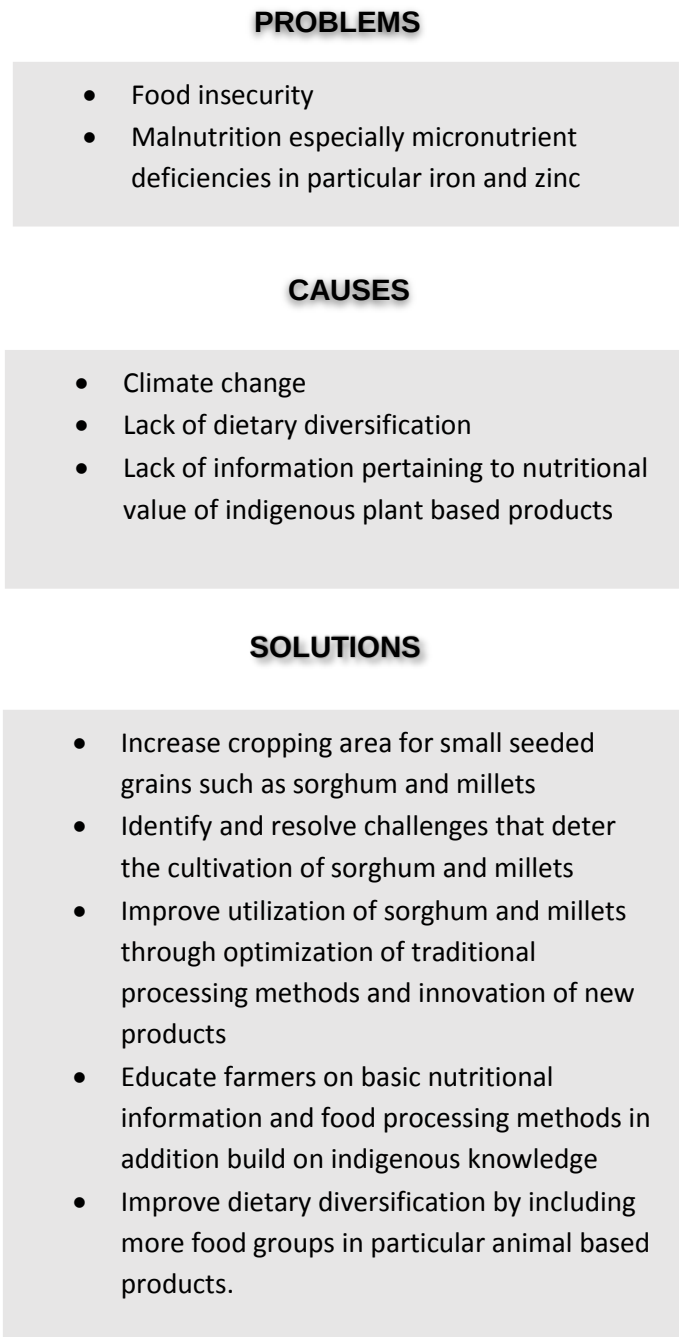


Figure 2.8: Schematic overview of an analysis of the food security problems in Ushe communal area and possible solutions

2.4.5 Conclusions

Small seeded grains are an important part of an African diet and their adoption should be highly encouraged in the face of increasing food insecurity and climatic risk. Government intervention is particularly important in setting up sustainable policies in vulnerable areas. Of particular importance in Ushe communal area is the adoption of all small seeded grains which can be aided by the introduction of good quality seeds resistant to bird attack and improved mechanization for easier processing. It is also important to establish and strengthen knowledge bases in rural communities to inculcate the production of safe and nutritious staple foods through optimized traditional methods. The traditional methods of fermentation and cooking used during the preparation of complementary porridges need to be investigated. Complementary porridges are important for children's nutrition hence their nutritional adequacy is of great importance.

Chapter 3:

Effect of traditional fermentation and cooking on mineral binders and subsequent iron and zinc bioaccessibility

Chapter 3 : Effect of household fermentation and cooking on mineral binders and subsequent iron and zinc bioaccessibility

3.1 Abstract

The aim of the present study was to explore the nutritional adequacy and the effect of traditional fermentation and cooking on mineral binders and subsequent bioaccessible iron and zinc contents of finger millet sour porridge consumed in Ushe communal area in Zimbabwe. Porridge products prepared from four traditional varieties of finger millet were collected and analyzed for proximate composition, mineral binders and subsequently *in vitro* iron and zinc bioaccessibility (proportion of dialyzable minerals, < 14 kDa). Fermentation and cooking caused a more than two fold increase in soluble phenolic compounds, condensed tannins and phenolic acids. However, a general decrease in the bound phenolic compounds was observed causing a reduction in the total phenolic compounds after fermentation and cooking. Compared to the raw materials, phenolic compounds and condensed tannins were reduced by up to 41% and 35% respectively while phytic acid was reduced by 22-54% in one variety only. Iron and zinc bioaccessibility was 6% and 13% respectively in the porridges and no improvement in bioaccessibility was observed as a result of processing as such bioaccessible iron and zinc contents only met less than 50% of the recommended daily intakes for children aged 1-3 years. A multidisciplinary approach is urgently needed to improve the iron and zinc contents and bioaccessibility of cereal based porridges from developing countries.

Keywords: Phenolic compounds, condensed tannins, phytic acid, finger millet, fermentation, cooking, iron, zinc, bioaccessibility

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AND

Gabaza, M., Shumoy, H., Louwagie, L., Muchuweti, M., Vandamme, P., Du Laing, G., Raes, K., (2017b). Traditional fermentation and cooking of finger millet: Implications on mineral binders and subsequent bioaccessibility. Journal of Food Composition and Analysis. Doi: 10.1016/j.jfca.2017.05.011.

3.2 Introduction

Cereals provide 77% of total caloric intake and substantially contribute to protein intake to the majority of the population belonging to resource poor population groups (FAO, 1999). Cereals are estimated to provide up to 60% of total iron and zinc dietary intake in Africa (Joy et al., 2014). The major cereals grown in Africa include maize, sorghum and millets which are mainly used for human consumption. Deficiencies of iron and zinc are extremely prevalent in developing countries affecting mostly young children. One of the many causes of iron and zinc deficiency is the consumption of monotonous cereal staples with a high amount of mineral binders such as phytic acid (PA), phenolic compounds (PC) and condensed tannins (CT) (Baye et al., 2015). Children are particularly vulnerable to iron and zinc deficiencies since many complementary porridges in Africa are based on fermented cereals with a drinkable consistency which means they have a low dry matter (dm) content of not more than 12%, making them dilute for their function (Nout, 1994). According to Gibson et al. (1998), the majority of the cereal based complementary foods consumed in low income countries could not meet the estimated daily requirements of iron and zinc assuming moderate bioavailability for children aged 9-11 months. More starkly, complementary foods mimicking the African cereal based complementary foods with high water content, could additionally not meet the estimated energy requirements.

The improvement of the energy content of fermented cereal porridges has been successfully done by fermenting a combination of cereals with legumes such as groundnuts, soybean and cowpeas and also optimizing the fermentation process through the use of starter cultures with the ability to hydrolyze starch so as to increase the dry matter content of the porridges (Songre-Ouattara et al., 2010). The increase in dry matter content of the porridges is not only important for macronutrient balance, but also increases the content of minerals assuming that same amount of porridge is consumed despite differences in dry matter content. Fermentation poses a great potential as it is commonly practiced to prepare complementary foods in Africa. The reduction of mineral binders (PA, PC and CT) has been observed e.g. during the preparation of *bensaalga* (Mouquet-Rivier et al., 2008), *ogi*/*uji* (Onyango et al., 2005), *mahewu* (Bvochora et al., 2005), *togwa* (Hellström et al., 2010) and fermented sorghum porridges (Kruger et al., 2012; Towo et al., 2006). However, whether this reduction in mineral binders is sufficient to cause a significant increase in mineral bioavailability is not clear. Many studies report an increase in solubility of iron and zinc after fermentation but solubility may not be a reliable indicator of bioavailability as it does not take into consideration the molecular size of the compounds which is critical for absorption (Etcheverry et al., 2012).

Sour porridge is a common type of fermented porridge used as a weaning and complementary food for children in Zimbabwe. It is almost entirely made from maize but can also be prepared from sorghum

and millets. Finger millet (*Eleusine coracana*) is widely used in the preparation of sour porridge in many Zimbabwean households and is a drought resistant crop with potential to increase the adaptive capacity of low income population groups to the effects of climate change (Rurinda et al., 2014a). Finger millet contains high amounts of phenolic compounds (PC), many of which have therapeutic benefits such as heart disease prevention, anticancer, antihypertensive and anti-inflammatory characteristics (Chandrasekara and Shahidi, 2010; Dykes and Rooney, 2006; Taylor et al., 2014; Van Hung, 2014). However, PC may behave like a double edged sword as on the one hand they exhibit positive health effects but on the other hand certain PC with catechol and galloyl groups may negatively affect iron and zinc absorption (Raes et al., 2014; Towo et al., 2006).

Fermentation and cooking are two processing methods widely used in the preparation of sour porridge in Zimbabwe and can induce important chemical changes such as the destruction of mineral binders. To our knowledge, the nutritional adequacy of sour porridge prepared from finger millet is not known and in particular the effect of fermentation and cooking on PA, PC and CT and its implications on iron and zinc bioaccessibility is not available. Additionally there is a paucity of information concerning the changes occurring to soluble and bound PC after fermentation and cooking of cereals, as well as regarding different finger millet varieties. Many studies report changes occurring to total contents of PC without making a distinction between soluble and bound PC (Chandrasekara and Shahidi, 2012; Hithamani and Srinivasan, 2014; Kayodé et al., 2013). This implies that the impact of processing conditions on the content and change in both soluble and bound PC fractions are not well known, thus their possible related effects towards health or interactions with other food components are unclear.

Therefore, the objective of this study was first to determine the effect of household fermentation and cooking on the mineral binders of household produced fermented finger millet porridges with particular focus on the soluble and bound PC. Secondly, to determine the proximate composition of the porridges and lastly to deduce the subsequent effect of the mineral binders on the iron and zinc bioaccessibility. Of importance in this study was the preparation of porridges under practical conditions. This study can thus provide baseline information that will guide future intervention strategies.

3.3 Materials and Methods

3.3.1 Materials

The finger millet flour and products were provided by women from the Hwedza communal area. Finger millet grains used were grown by the women in the period 2013-2014 and were traditional varieties commonly grown and consumed in this area. Two red and two white finger millet varieties were used

in preparing the products as such each group was identified according to the variety of finger millet prepared i.e. RV1-red variety 1, RV2-red variety 2, WV1-white variety 1 and WV2-white variety 2. The peanut butter was also provided by the women as a product of groundnuts harvested in the same period. Methanol, sodium hydroxide, sodium carbonate and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu's reagent, phytic acid dodecasodium salt, 2,2' bipyridine and thioglycolic acid were purchased from ChemLab (Zedelgem, Belgium). Gallic acid, catechin, *p*-coumaric acid, ferulic acid, syringic acid, sinapic acid, vanillic acid, protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid, cinnamic acid, α-amylase from porcine pancreas (Type VI-B, > 10 units/mg solid), pepsin from porcine gastric mucosa lyophilized powder (3200-4500 units/mg protein), pancreatin from porcine pancreas (8xUSP, P7545), dialysis bags (99.99% retention seamless cellulose tubing, width 32 mm, height 30 m, MW cut off 12-14 kDa) and bile from porcine bile extract were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). HPLC-grade methanol, HPLC-grade water and trifluoroacetic acid were purchased from VWR (Leuven, Belgium). ICP multi-element standard solution IV was purchased from Merck (Germany).

3.3.2 Methods

3.3.2.1 Sample preparation

Ushe communal area in Hwedza district of Zimbabwe was selected as the study site as it is located in a region that had the highest level of anaemia and stunting (Central Statistics Office, 2011). Two learning meetings were carried out in June 2014 in order to understand the utilization of small seeded grains in this area. Finger millet emerged as the small seeded grain that is mainly cultivated and also used to make porridge. The basic method of preparing porridge was identified and differed in several aspects such as level of ingredients used, seed coat color of the grains i.e. red or white variety and duration of fermentation. Women from 24 households from Ushe communal area were organized into four groups and tasked with the preparation of finger millet products using their traditional preparation process as revealed during the learning meetings. The grains were carefully cleaned and milled at the local commercial mill (using commercial hammer mill) to a particle size of 1 mm as normally practiced. The women prepared two types of fermented slurries i.e. spontaneously fermented slurry (SFS) and backslopped fermented slurry (BFS). Spontaneous fermentation is initiated through the action of the natural microorganisms that are present on the raw materials and utensils used, while backslopped fermentation is initiated by the inoculation of the raw material with a small quantity of a previously performed successful fermentation. In addition to the fermented slurries, four types of fermented porridges were also prepared i.e. spontaneously fermented porridge (SFP), backslopped fermented porridge (BFP), spontaneously fermented porridge with peanut butter (SFP_p)

and backslopped fermented porridge with peanut butter (BFP_p). The recipes used in the preparation of products were recorded accurately and with as much detail as possible and are shown in **Table 3.1**. The fermentation process was done in a thatched kitchen with approximate temperature of 23-25^{o}C. An aliquot of flour was measured into a fermentation vessel which could be a plastic or metal container followed by carefully adding a measured aliquot of water. The vessel was tightly closed and spontaneous or backslopped fermentation allowed to proceed for 24-36 hours. Successful fermentation was identified by a characteristic frothing of the fermented slurry at the surface and a typical fermented aroma (measured pH of 4 - 4.5). The fermented slurry was then cooked for 15-20 minutes, with or without extra added water, to make the porridge. **Table 3.1** shows the type of products and amount of ingredients used by each group, as well as the used preparation conditions.

3.3.2.2 Sample collection

Fermented slurries and porridge samples were collected in aliquots of 50 mL in sterile falcon tubes between July 1 and 12, 2014. The sampling was done twice within a time frame of two weeks. A total amount of 150 g was collected per sample. Samples were always transported under cooled conditions and immediately frozen at -80^{o}C. Samples were transported to Belgium under dry ice and stored at -20^{o}C. Flour of the different varieties was also collected and stored at -20^{o}C at the Laboratory of Food Microbiology and Biotechnology, Ghent University, Campus Kortrijk, Belgium.

3.3.2.3 Extraction of soluble phenolic compounds

Extraction of soluble PC was based on Gonzales et al. (2014). Briefly, 5 g of porridge samples and fermented slurries and 2 g of flour samples were added with 15 ml absolute methanol in 50 ml falcon tubes. The mixture was homogenized using an ultra turrax (IKA T18 Basic) at 10000 rpm for 45 s and placed on ice for 15 min. thereafter. To separate the soluble extract from the residue, the mixture was centrifuged (Z 300 K, Hermle Labortechnik, GmbH, Germany) at 4000 rpm, 15 min. at 4^{o}C. Re-extraction was done using 80% methanol following the same procedure. The collected supernatants were pooled and filled to 25 ml with 80% methanol. The extract was transferred to storage bottles covered with aluminum foil to protect from sunlight and stored at -20^{o}C until further analysis. The residue was dried overnight at room temperature and bound PC were extracted from it the next day.

Table 3.1: Levels of ingredients and processing parameters used in the preparation of finger millet fermented slurries and porridges

Variety Products	RV1	RV2	WV1	WV2
SFS	100 g flour 300 mL water 24 hrs. fermentation 25-30^{o}C	100 g flour 200 mL water 24 -30 hrs. fermentation 25-30^{o}C	100 g flour 300 mL water 24 hrs. fermentation 25-30^{o}C	100 g flour 400 mL water 36 hrs. fermentation 25-30^{o}C
SFP	Extra 200 mL water, 80-100^{o}C, 20 min.	Extra 300 mL water 80-100^{o}C, 20 min.	No extra water added 80-100^{o}C, 15 min.	Extra 400 mL water 80-100^{o}C, 20 min.
SFP_p	Same as SFP + 40 g peanut butter	Same as SFP + 45 g peanut butter	Same as SFP + 35 g peanut butter	Same as SFP + 40 g peanut butter
BFS	100 g flour 300 mL water 50 g prefermented slurry 24 hrs. fermentation 25-30^{o}C	100 g flour 200 mL water 50 g prefermented slurry 24 -30hrs fermentation 25-30^{o}C	100 g flour 300 mL water 50 g prefermented slurry 24 hrs. fermentation 25-30^{o}C	100 g flour 400 mL water 100 g prefermented slurry 36 hrs. fermentation 25-30^{o}C
BFP	Extra 200 mL water, 80-100^{o}C, 20 min.	Extra 350 mL water, 80-100^{o}C, 20 min.	No extra water added, 80-100^{o}C, 15 min.	Extra 350 mL water, 80-100^{o}C, 20 min.
BFP_p	Same as BFP + 40 g peanut butter	Same as BFP + 45 g peanut butter	Same as BFP + 35 g peanut butter	Same as BFP + 40 g peanut butter

RV1: Red variety 1, RV2: Red variety 2, WV1: White variety 1, WV2: White variety 2, SFS: Spontaneously fermented slurry, SFP: Porridge from SFS, SFP_p: porridge from SFS with peanut butter, BFS: backslopped fermented slurry, BFP: porridge from BFS, BFP_p: porridge from BFS with peanut butter.

3.3.2.4 Extraction of bound phenolic compounds

Bound PC were extracted according to method described by Gonzales et al. (2014). The dried residue, obtained after extraction of the soluble PC (0.1 g) was placed in 25 ml screw-capped falcon tubes previously flushed with nitrogen and hydrolyzed with 2 ml NaOH (2M) in a sonicated water bath (UP 400 S, dr.Hielscher, GmbH, Germany) at 60^{o}C for 30 min. The mixture was neutralized with 4 ml HCl (2M) and extracted twice with 0.1 % formic acid in absolute methanol for 2 min. under vortex. Supernatants were always separated by centrifugation at 4000 g for 15 minutes and 4^{o}C. The supernatants were pooled and filled to 25 ml using 80 % methanol and stored in storage bottles at -20^{o}C covered in aluminum foil.

3.3.2.5 Determination of total phenolic compounds

The total PC were determined on both the soluble and bound fractions according to the Folin-Ciocalteu method as described by Singleton and Rossi (1965). Briefly, extracts from soluble and bound fraction (1 ml) were added to test tubes with 1 ml deionized water following by 0.5 ml ten times diluted Folin-Ciocalteu's reagent. After 5 min., 1.5 ml of 20 % sodium carbonate and 1 ml deionized water was added and the mixture was allowed to stand for 2 h in the dark after which the absorbance was read at 760 nm. The phenolic contents for each fraction was calculated based on a standard curve generated from gallic acid and expressed as mg gallic acid equivalents (GAE)/100 g dm.

3.3.2.6 Determination of condensed tannins

Proanthocyanidins or CT were analyzed according to the method described by Price et al. (1978). Bound and soluble extracts and standards (1 ml) were added to test tubes followed by 5 ml of vanillin reagent (50:50 mixture of 5% vanillin and 24 % HCl w/v) and incubated for exactly 20 min. in a water bath at 30^{o}C. A second set of control tubes was prepared by adding 1 ml of sample or standards followed by 12 % HCl, and incubated under the same conditions after which the absorbance was read at 500 nm. The final absorbance was determined by subtracting the absorbance of the sample control from the corresponding vanillin-containing sample. The CT for each fraction was calculated based on a standard curve generated from catechin and expressed as mg catechin equivalents (CE)/100 g dm.

3.3.2.7 Identification of phenolic compounds by HPLC

Identification of PC was done following the method described by Wen et al. (2005). Soluble and bound sample extracts were dried under nitrogen, and redissolved in 500 µL of MeOH:water:trifluoroacetic acid (TFA) (50: 50: 0.1) and filtered. An internal standard of o-coumaric acid was added to the samples and the samples were injected directly into the HPLC system. The separation of phenolic acids was carried out with Agilent HPLC equipped with column (Alltima^{TM} –Column 18 5u (4.6 mm × 150 mm), GRACE, Deerfield, USA). The mobile phase consisted of solvent mixture A (0.02 % TFA in water) and solvent mixture B (0.02 % TFA in methanol). The following program was used for gradient elution: 0-5 min. (25 % B), 5- 10 min. (25-30% B), 10-16 min. (30-45 % B), 16-18 min. (45% B), 18-25 min. (45-80% B), 25-30 min. (80% B), 30-40 min. (80-25% B). Samples were injected at a volume of 10 µL. The DAD detection was set at four wavelengths namely 254 nm (for identification of protocatechuic and vanillic acid), 275 nm (gallic, syringic, cinnamic, catechin and internal standard), 305 nm (p-coumaric and salicylic acid) and 320 nm (caffeic, ferulic, sinapic and rosmarinic acid). Quantification of identified phenolic acids was done using corresponding regression equation and values are expressed as µg/g dm.

3.3.2.8 Phytic acid analysis

PA content was analyzed spectrophotometrically using the Haug and Lantzsch method as described by Reichwald and Hatzack (2008). Samples of flour, fermented slurries and porridges (0.1 g) were placed in test tubes and PA was extracted with HCl on a shaking water bath at 100°C for 45 min. The supernatant was collected and diluted five times in deionized water. Aliquots of the diluted supernatant and standards were transferred to new set of tubes and ferric solution was added after which tubes were put back on the shaking water bath for another 45 min. The tubes were then cooled on ice for 15 min. and 600 µL of the supernatant and standards were added to microcuvettes followed by 800 µL of complexing reagent consisting of 2.5 g of 2,2' bipyridine and 325 µL thioglycolic acid dissolved in 250 mL HCl (0.2M). The absorbance was measured at 540 nm and results were expressed as mg/100g dm. PA/mineral molar ratios were calculated using molecular weights of PA (660 g/mol), iron (56 g/mol) and zinc (65 g/mol).

3.3.2.9 Proximate analysis of porridges

For the proximate analysis, which was done on the porridges only, dry matter, protein, fat and ash content was determined according to ISO 1442-1973, ISO 937-1978, ISO 1444-1973, and AACC (2000) respectively. The energy content (kcal/100 g fresh weight) of the porridge was calculated as follows:

$$Energy\ content\ in\ kcal/100g = (\%CHO * 4\ kcal + \%Protein * 4\ kcal + \%Fat * 9\ kcal\ ) + 5\%\ sugar * 4\ kcal$$

*An estimated 5 g of sugar was added to 100 g serving of porridge before consumption.

3.3.2.10 Analysis of iron and zinc content

Iron and zinc contents were analyzed by ICP-OES following the method by Ashoka et al. (2009). Briefly, 2 g of freeze dried sample was completely carbonized, and placed in a muffle oven at 550°C overnight. After cooling the ash was dissolved in 50% nitric acid, filtered and iron and zinc contents were measured by ICP-OES at wavelengths of 259 nm and 213 nm, respectively. Results are expressed as mg/100 g dm.

3.3.2.11 *In vitro* digestion

In vitro digestion of flour, fermented slurries and porridges was done using the standardized static *in vitro* digestion method outlined by (Minekus et al., 2014). This consensus and harmonized static

digestion method for food which is based on physiologically relevant conditions that can be applied at various endpoints was proposed by the COST Infogest network for the production of more comparable data in the future (https://www.cost-infogest.eu/). Fresh sample (flour, fermented slurries and porridges) equal to a dry matter content of 1.5 g was weighed into a digestion flask and mixed with 5 mL simulated salivary fluid (KCl – 15.1 mmol/L, KH_2PO_4 – 3.7 mmol/L, $NaHCO_3$ – 13.6 mmol/L, $MgCl_2$ – 0.15 mmol/L, $(NH_4)_2CO_3$ – 0.06 mmol/L, pH 7) followed by mincing using a kitchen electrical mincer for the porridges only. Aliquot of 0.5 mL porcine α-amylase (1500 U/mL) was added followed by 25 µL of 0.3 M $CaCl_2$ and 975 µL of water. The mixture was thoroughly homogenized. Then pH was corrected to 7 followed by incubation for 2 min. in a shaking water bath at 37°C. To the resultant oral digested mixture, 7.5 mL of simulated gastric fluid (KCl – 6.9 mmol/L, KH_2PO_4 – 0.9 mmol/L, $NaHCO_3$ – 25 mmol/L, NaCl – 47.2 mmol/L, $MgCl_2$ – 0.1 mmol/L, $(NH_4)_2CO_3$ – 0.5 mmol/L, pH 3), 1.6 mL of porcine pepsin (25000 U/mL), 5 µL of $CaCl_2$ and 0.695 µL water was added, and the pH was corrected to 3, with subsequent incubation in a water bath at 37°C for 2 h. After 90 min. of gastric digestion, dialysis bags of 15.5 cm length containing a mixture of 5.5 mL 0.9% NaCl and 5.5 mL 0.5 M of $NaHCO_3$ were added to each digestion vessel and the digestion was continued for another 30 min. After 2 h of gastric digestion, 11 mL of simulated intestinal fluid (KCl – 6.8 mmol/L, KH_2PO_4 – 0.8 mmol/L, $NaHCO_3$ – 85 mmol/L, NaCl – 38.4 mmol/L, $MgCl_2$ – 0.33 mmol/L, pH 7) was added followed by 5.0 mL pancreatin solution (800 U/mL), 2.5 mL fresh bile extract, 40 µL 0.3 M $CaCl_2$ and 1.31 mL of water. The pH was corrected to 7, and digestion was continued by incubation under the same conditions for another 2 h. Dialysis bags were removed from the digestion vessels and washed with distilled water. The dialysate, (D), which is the fluid in the dialysis bags and also considered as the bioaccessible fraction (Bioaccessibility was defined as the proportion of minerals able to through a dialysis membrane of 12-14 kDa molecular weight cut-off), was transferred to new tubes while the digested mixture was centrifuged and separated into the soluble non-dialyzable (SND) and insoluble fraction or pellet, (P). The three fractions namely: D, SND and P were ashed and iron and zinc contents were analyzed by ICP-OES according to the protocol outlined previously. The D, SND and P mineral fractions were calculated based on the total sum of mineral recovered after digestion using the formulas below:

$$Bioaccessible\ Fe\ or\ Zn\% = \frac{Fe\ or\ Zn\ in\ dialyzable\ fraction\ (mg/100g\ dm)}{(D + SND + P) Fe\ or\ Zn\ (mg/100g\ dm)} * 100\%$$

$$SND\ Fe\ or\ Zn\% = \frac{Fe\ or\ Zn\ in\ SND\ fraction\ (mg/100g\ dm)}{(D + SND + P)\ Fe\ or\ Zn(mg/100g\ dm)} * 100$$

3.3.2.12 Statistical Analysis

All statistical analysis were done using IBM SPSS software version 20. One way ANOVA was done to check for differences among treatments within each variety and if needed, comparison of means was done using Tukey's post-hoc analysis ($p < 0.05$). Values in tables are shown as mean ± standard deviation of two independent samples.

3.4 Results

3.4.1 Soluble and bound phenolic compounds and condensed tannins of finger millet flour

Figure 3.1 shows the soluble and bound PC found in red and white finger millet flour from Ushe communal area, Zimbabwe. The total content of PC of the red and white finger millet ranged from 1108-1330 and 334-359 mg GAE/100g dm, respectively. The bound fraction accounted for approximately 95% of the total PC for both red and white millet samples. The CT in the white varieties could not be detected (limit of detection for CT - 0.008 mg/L). Higher amounts of CT in the bound fraction (89-94% of the total CT content) than in the soluble fraction was also observed, due to the association of CT with cell walls **(Figure 3.2).** The white varieties of finger millet contained lower amounts of PC than the red varieties.

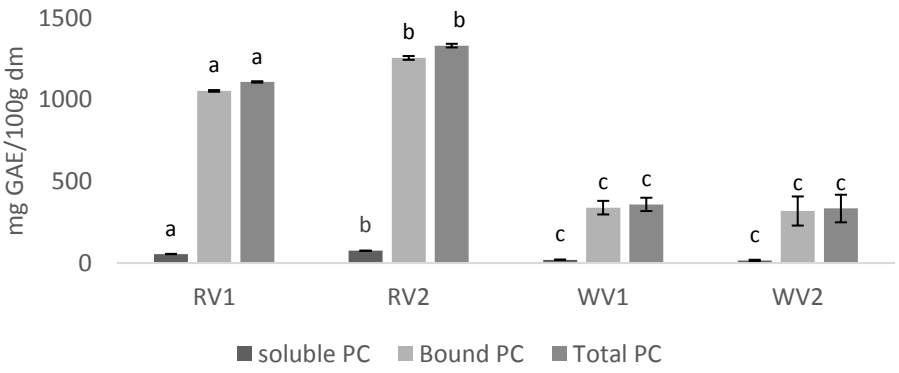

Figure 3.1: Soluble and bound phenolic compounds of finger millet flour

RV1: Red variety 1, RV2: Red variety 2, WV1: White variety 1, WV2: White variety 2, PC: phenolic compounds, GAE: gallic acid equivalents. Bars with the same color with different letters are significantly different ($p < 0.05$), n=2.

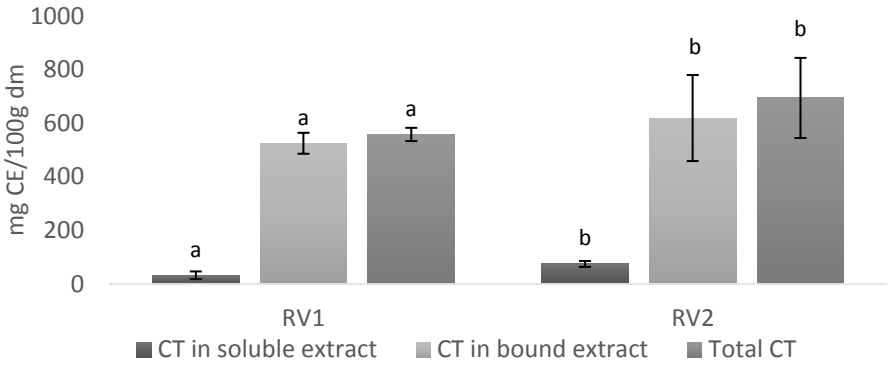

Figure 3.2: Content of condensed tannins in red varieties of finger millet flour

RV1: Red variety 1, RV2: Red variety 2, CT: condensed tannins, CE: catechin equivalents. Bars with the same color with different letters are significantly different ($p < 0.05$), n=2.

3.4.2 Effect of fermentation and cooking on soluble and bound phenolic compounds and condensed tannins of finger millet

The soluble, bound and total PC and CT of the finger millet products from red and white varieties are shown in **Tables 3.2 and 3.3**. After both spontaneous and backslopped fermentation, there was a general reduction in the content of bound and total PC and CT while there was a significant increase in the soluble PC in all the varieties ($p < 0.05$). This shows that despite different household processing conditions, in general, fermentation reduced the content of bound and total PC and increased the content of soluble PC. Total PC were reduced by 6.4-31% while bound PC were reduced by 17-37% for the spontaneous fermentations while total PC and bound PC were reduced by 9-39% and 17-47% respectively for the backslopped fermentations. The lowest reductions in bound and total PC were observed for the white varieties regardless of type of fermentation. On the other hand, soluble PC were increased by 16-167% for the spontaneous fermentation while an increase of 48-138% was observed for the backslopped fermentations. In terms of CT, a decrease of 32-56% and 43-59% was observed for total and bound CT respectively for both spontaneous and backslopped fermentations while an increase of up to 89% was seen for the soluble CT except for BFS of RV1 which showed no change. After fermentation, cooking of fermented slurries followed to make the porridge. In comparison with the fermented slurries, soluble PC and CT significantly increased in all porridges despite type of fermentation by up to 186% for soluble PC and up to 263% for soluble CT. For both spontaneous and backslopped fermentations, the bound PC increased by 22-38% for the red varieties while they decreased by 26-44% for the white varieties. In general, cooking after fermentation caused an increase in total PC for the red varieties while there was a decrease for the white varieties. In terms of the CT of the porridges, no significant change was observed for the bound and total CT compared

to both spontaneous and backslopped fermented slurries. Addition of peanut butter generally caused no effect on the soluble, bound and total PC for all porridges compared with the porridges without peanut butter although some slight changes were observed for RV2 and WV2. Pertaining to CT, an increase in soluble CT was observed for backslopped fermented slurry of RV1 and RV2 and spontaneously fermented slurry of RV2, in comparison with the porridges without peanut butter while no change was generally observed for the bound and total CT. The overall effect of cooking combined with fermentation caused more than two times increase in both the soluble PC and CT of the cooked porridges for both types of fermentations. In addition, bound PC, CT and total PC and CT were also mostly reduced.

3.4.3 Profile of the phenolic compounds in finger millet flour

Seven phenolic acids i.e. cinnamic acid, syringic acid, *p*-coumaric acid, salicylic acid, sinapic acid, ferulic acid, protocatechuic acid, caffeic acid and one flavonoid namely catechin were identified in the different varieties of finger millet **(Table 3.4 and 3.5)**. Caffeic acid was only found in the bound fraction. Catechin was the only flavonoid identified in the soluble fraction where its peak occupied more than 50% of the total area in soluble extracts. The major phenolic acids identified were ferulic acid, sinapic acid and salicylic acid. Ferulic acid was the most abundant phenolic acid in the bound fractions of both the white and red varieties with values ranging from 107-193 µg/g. Sinapic acid was higher in the white varieties (241-330 µg/g) than in the red varieties (49.1-110 µg/g) and 37-59% was found in the bound fraction. Sinapic acid appeared to be the major phenolic acid in white variety and also appeared to be the dominant soluble phenolic acid in all the varieties. Salicylic acid was also found in high amounts with up to 195 µg/g in the bound fraction of WV2 and 75-98% existing in the bound fraction. Several peaks in both soluble and bound fractions could not be identified (approximately 20-30% of the total peak area).

3.4.4 Effect of fermentation and cooking on soluble and bound individual phenolic compounds of finger millet

In all varieties, an increase of all individual soluble phenolic acids was observed after fermentation and cooking while an inconsistent trend was seen for bound phenolic acids **(Table 3.4 and 3.5)**. Catechin increased more than four times after fermentation and cooking with the highest increase in the red varieties. After cooking, levels of phenolic acids increased in bound fraction except for samples from WV1 where all bound phenolic acids decreased after cooking. No clear effect for both soluble and bound phenolic compounds was observed in all varieties after the addition of peanut butter.

Table 3.2: Phenolic compounds in finger millet flour, fermented slurries and porridges

	RV1			RV2			WV1			WV2		
	Soluble	**Bound**	**Total**	**Soluble**	**Bound**	**Total**	**Soluble**	**Bound**	**Total**	**Soluble**	**Bound**	**Total**
Flour	55.1±0.1^{a}	1053±5^{d}	1108±4^{d}	75.1±4.7^{a}	1255±89^{b,c}	1330±84^{a}	20.1±0.6^{a}	339±12^{c}	359±11	15.3±0.9^{a}	318±42^{c}	334±41^{d}
SFS	107±2^{b}	662±71^{b}	769±70^{b,c}	87.2±6.2^{a,b}	895±100^{a}	982±98^{c}	53.7±4.0^{b,c}	281±24^{b,c}	336±21	21.7±2.1^{a}	215±13^{b}	237±12^{a,b}
SFP	142±24^{c}	805±21^{c}	947±49^{c}	249±5^{b,e}	1200±5^{b,c}	1450±1^{a}	71.6±10.1^{d}	156±85^{b,c}	255±91	51.4±8.6^{b}	159±14^{a}	213±21^{a}
SFP_p	118±18^{b,c}	760±39^{b,c}	878±11^{b,c}	131±22^{c,d}	942±23^{a,b}	1073±41^{b,c}	69.8±5.8^{d}	198±29^{a,b}	277±32	56.4±5.0^{b}	235±13^{b}	292±18^{c}
BFS	114±5^{b,c}	559±52^{a}	673±56^{a}	111±13^{b,c}	796±171^{a}	907±176^{c}	47.9±7.7^{b}	281±8^{b,c}	326±13	25.4±3.0^{a}	236±18^{b}	260±19^{b,c}
BFP	169±18^{c}	781±51^{c}	950±59^{c}	233±12^{e}	1089±45^{a,b}	1322±40^{a,b}	65.7±7.9^{c,d}	160±70^{a}	226±99	50.9±6.1^{b}	147±16^{a}	198±11^{a}
BFP_p	124±2^{b,c}	759±79^{b,c}	883±62^{b,c}	155±17^{d}	850±176^{a}	1004±190^{c}	61.3±7.6^{b,c}	268±14^{b,c}	326±15	48.5±9.5^{b}	221±8^{b}	270±17^{b,c}
p values	<0.001	<0.001	0.035	<0.001	0.001	<0.001	<0.001	0.001	0.126	<0.001	<0.001	<0.001

RV1: red variety 1, RV2: red variety 2, WV1: white variety 1, WV2: white variety 2, SFS: spontaneously fermented slurry, SFP: porridge from SFS, SFP_p: porridge from SFS with peanut butter, BFS: backslopped fermented slurry, BFP: porridge from BFS, BFP_p: porridge from BFS with peanut butter. All values expressed as mg GAE/100 g dm. Values followed by different superscript letters in the same column are significantly different ($p < 0.05$), n=2.

Table 3.3: Condensed tannins in finger millet flour, fermented slurries and porridges

	RV1			RV2		
	Soluble	**Bound**	**Total**	**Soluble**	**Bound**	**Total**
Flour	32.8±14.4^{a}	525±39^{a}	558±25^{b}	75±11^{a}	619±161^{a}	694±150^{c}
SFS	44.1±26.3^{a}	274±96^{b}	264±98^{a}	116±15^{a,b}	352±79^{b}	468±91^{a,b}
SFP	77.8±21.8^{a,b}	335±65^{b}	413±43^{a,b}	223±12^{c}	224±72^{b}	448±60^{a,b}
SFP_P	64.8±33.4^{a,b}	171±55^{b}	236±55^{a}	73.2±4.6^{a}	257±48^{b}	330±44^{a}
BFS	34.7±20.5^{a}	213±33^{b}	247±69^{a}	142±39^{b}	404±22^{b}	546±25^{b,c}
BFP	126±34^{c}	293±80^{b}	418±72^{a,b}	218±14^{c}	271±97^{b}	489±98^{a,b}
BFP_p	29.8±10.2^{a}	221±85^{b}	250±60^{a}	91.6±10.8^{a}	300±64^{b}	392±58^{a,b}
p values	0.008	0.003	0.001	<0001	0.001	0.001

RV1: red variety 1, RV2: red variety 2, SFS: spontaneously fermented slurry, SFP: porridge from SFS, SFP_p: porridge from SFS with peanut butter, BFS: backslopped fermented slurry, BFP: porridge from BFS, BFP_p: porridge from BFS with peanut butter. All values expressed as mg CE/100g dm. Values followed by different superscript letters in the same column are significantly different ($p < 0.05$), n=2.

Table 3.4: Individual phenolic compounds in soluble and bound extracts of finger millet flour, fermented slurries and porridges of red varieties

	Soluble				Bound			
PC	**Flour**	**SFS**	**SFP**	**SFP_p**	**Flour**	**SFS**	**SFP**	**SFP_p**
RV1								
p-coumaric acid	4.45±1.92	11.7±1.6	15.5±1.8	28.4±0.5	16.1±0.5	26.8±11.9	24.0±2.1	38.9±14.8
Salicylic acid	12.4±5.6	36.9±7.5	48.1±5.4	35.4±4.3	36.8±4.1	49.2±10.3	59.2±4.9	61.5±13.6
Sinapic acid	59.7±20.9	113±11	122±6	223±44	50.1±21.2	115±21	297±68	nv
Ferulic acid	0.91±0.13	2.61±0.02	3.01±0.75	9.71±3.97	107±18	147±30	183±2	192±23
Protocatechuic acid	15.8±5.7	44.3±6.0	nd	nd	33.9±1.5	30.7±5.7	41.0±3.6	42.6±5.4
Caffeic acid	nd	nd	nd	nd	12.6±1.3	15.7±0.6	17.9±0.2	18.5±3.1
Catechin	178±63	644±52	777±120	647±158	nd	nd	nd	nd
RV2								
p-coumaric acid	1.51±0.38	2.23±0.48	3.32±0.52	26.8±2.1	28.8±10.1	21.0±0.3	27.9±4.3	91.9±0.1
Salicylic acid	2.10±0.21	2.23±1.19	7.33±4.46	14.2±7.6	159±4	149±19	147±7	121±12
Sinapic acid	31.1±13.8	152±76	195±33	260±12	18±12	nv	nv	nv
Ferulic acid	0.59±0.11	2.30±0.78	2.21±0.25	5.94±1.56	176±55	144±19	140±15	140±3
Protocatechuic acid	2.75±0.88	5.52±2.40	13.5±2.9	5.53±2.57	80.8±28.1	48.2±8.8	54.4±3.5	61.3±5.9
Caffeic acid	nd	nd	nd	nd	25.7±6.8	22.6±2.4	25.0±3.0	29.0±0.7
Catechin	224±48	555±174	891±168	692±168	nd	nd	nd	nd

RV1: red variety 1, RV2: red variety 2, SFS: spontaneously fermented slurry, SFP: porridge from SFS, SFP_p: porridge from SFS, nv: no value, nd: not detected. Limit of detection for caffeic acid, protocatechuic acid and catechin was 0.52, 0.35 and 2.05 mg/L respectively. All values are expressed as µg/g dm, n=2.

Table 3.5: Individual phenolic compounds in soluble and bound extracts of finger millet flour, fermented slurries and porridges of white varieties

	Soluble				Bound			
PC	**Flour**	**SFS**	**SFP**	**SFP_p**	**Flour**	**SFS**	**SFP**	**SFP_p**
WV1								
p-coumaric acid	9.16±1.63	22.5±5.8	24.4±7.9	42.1±3.1	11.9±3.1	9.45±1.86	19.6±3.1	21.9±9.9
Salicylic acid	20.8±3.6	57.2±7.2	51.9±9.1	52.0±7.8	195±22	381±9	250±21	113±26
Sinapic acid	193±33	209±130	198±67	201±3	137±32	451±68	274±49	nv
Ferulic acid	1.22±0.42	5.14±0.92	5.01±1.28	4.42±0.50	123±25	195±4	171±36	67.8±20.3
Protocatechuic acid	4.31±0.56	nd	9.97±0.34	6.89±0.99	3.92±0.91	nd	nd	nd
Caffeic acid	nd	nd	nd	nd	18.5±0.7	25.7±3.1	21.4±3.7	11.7±1.9
Catechin	69.6±6.7	286±160	276±79	225±15	nd	nd	nd	nd
WV2								
p-coumaric acid	3.78±0.39	4.56±0.81	10.5±5.4	6.68±2.49	25.9±3.6	3.35±1.31	16.0±1.4	42.6±3.3
Salicylic acid	5.13±0.25	22.1±10.6	21.3±11.5	20.7±3.2	191±57	82.4±33.1	304±32	183±15
Sinapic acid	99.3±0.4	103±51	152±82	115±45	142±14	nv	159±59	nv
Ferulic acid	1.56±0.09	3.29±1.42	2.85±0.81	4.25±1.03	193±13	22.9±5.8	187±48	154±16
Protocatechuic acid	nd	nd	nd	nd	nd	nd	nd	nd
Caffeic acid	nd	nd	nd	nd	39.8±5.4	6.01±3.07	35.9±6.6	37.1±7.7
Catechin	43.1±1.6	71.6±37.8	182±50	190±45	nd	nd	nd	nd

WV1: white variety 1, WV2: white variety 2, SFS: spontaneously fermented slurry, SFP: porridge from SFS, SFP_p: porridge from SFS, nv: no value, nd: not detected. Limit of detection for caffeic acid, protocatechuic acid and catechin was 0.52, 0.35 and 2.05 mg/L respectively. All values are expressed as µg/g dm, n=2.

3.4.5 Phytic acid content during preparation of sour porridge

Table 3.6 show the changes that occurred to PA during processing of finger millet into sour porridge. The PA content of the flours ranged between 739-1047 mg/100g dm and the white varieties had higher PA levels than the red varieties. After fermentation, no change in PA was observed for RV2 while a slight increase was observed for RV1 and WV1 ($p < 0.05$). There was a decrease of PA in WV2 after both spontaneous (22%) and backslopped fermentation (54%). In general, cooking increased PA across all samples. PA/Fe ratios ranged from 15.1-81.4 while for zinc ratios of 47.4–103 were calculated.

3.4.6 Proximate composition of porridges

No differences were observed based on fermentation technique used thus only the results for the spontaneous fermented and cooked products are shown. The average dm content for SFP was 11% and increased to an average of 17.5% after addition of peanut butter **(Table 3.7).** The carbohydrate content ranged from 9.28-12.8 g/100 g fresh weight (fw) SFP and was slightly higher after the addition of peanut butter. The latter increased the protein content from 0.87-1.03 g/100 g fw to 1.78-3.08 g/100 g fw while the amount of lipids increased from 0.14-0.19 g/100 g fw to 2.31-3.87 g/100 g fw. Ash content of porridges also increased after adding peanut butter from 0.27-0.41 g/100 g fw to 0.42-0.64 g/100 g fw. Of particular interest was the energy content which averaged 264 kJ/100 g fw and 427 kJ/100 g fw for SFP and SFP_p, respectively. There was a slight overestimation of about 5% in the energy density as no distinction was made between available carbohydrates and fibers.

3.4.7 Iron and zinc contents during preparation of sour porridge

The effect of processing on iron and zinc contents during the production of porridges is shown in **Table 3.8.** The iron content of the millet flour ranged from 0.78-2.05 mg/100 g dm while the zinc content ranged from 0.94-1.28 mg/100 g dm. The effect of fermentation and cooking caused inconsistent effects on the mineral contents. Comparing the flour with the fermented samples, there was an increase in iron from 11-139% and comparing with cooked samples i.e. SFP, BFP, SFP_p and BFP_p, an increase in iron content of up to 100% was observed. With respect to zinc content, slight increases and in some cases no change was observed in fermented and in cooked samples in comparison with the flour. The highest increases of zinc content of up to 66% were observed in the cooked porridges with peanut butter i.e. SFP_p and BFP_p.

3.4.8 Effect of fermentation and cooking on *in vitro* iron and zinc bioaccessibility

Figure 3.3 shows the results for the *in vitro* bioaccessibility of iron and zinc for RV2 as measured by dialyzability. *In vitro* iron bioaccessibility was 7.7%, 5.7% and 6.1% for flour, SFS and SFP, respectively while for zinc it was 12.7% (flour), 9.7% (SFS) and 13.2% (SFP). No improvement was observed for both iron and zinc *in vitro* bioaccessibility after processing. In addition, no effect of variety was observed on both iron and zinc bioaccessibility. The total soluble iron (SND + D%) was approximately 25% for flour, SFS and SFP. In contrast, the total soluble zinc was on average 49% for flour and SFS and increased to 72% for the SFP. However, the increase in total soluble zinc did not result in increased zinc bioaccessibility. Amount of bioaccessible iron was 0.065, 0.056 and 0.066 mg/100 g dm for flour, SFS and SFP, respectively, while for zinc it was 0.13, 0.12 and 0.18 mg/100 g dm for flour, SFS and SFP, respectively. In addition, no difference in bioaccessibility was observed as a result of differences in fermentation techniques and variety.

Table 3.6: Levels of PA, PA/Fe, PA/Zn of finger millet flour, fermented slurries and porridges

	PA				PA/Fe				PA/Zn			
Product	**RV1**	**RV2**	**WV1**	**WV2**	**RV1**	**RV2**	**WV1**	**WV2**	**RV1**	**RV2**	**WV1**	**WV2**
Flour	739±70^{a,b}	759±49^{a}	899±23^{a}	1047±77^{b}	46.0	81.4	59.1	40.4	78.0	81.1	90.9	80.6
SFS	993±74^{c}	909±116^{a}	1261±170^{b}	815±339^{a,b}	29.4	72.2	46.7	30.1	84.7	71.5	87.3	81.5
SFP	725±64^{a,b}	845±45^{a}	1127±125^{a,b}	1065±46^{b}	27.8	61.8	41.8	32.5	63.9	67.0	102	68.8
SFP_p	896±93^{a}	726±100^{a}	1074±37^{a,b}	1003±37^{b}	24.8	46.5	39.4	44.9	53.6	57.5	73.7	66.5
BFS	919±22^{c}	779±152^{a}	1101±207^{a,b}	478±268^{c}	24.3	75.8	45.4	15.1	70.5	55.4	103	47.4
BFP	715±75^{b,c}	932±32^{a}	994±99^{a,b}	1098±100^{b}	30.7	47.7	38.6	32.7	67.7	53.0	102	61.0
BFP_p	937±79^{c}	766±105^{a}	880±87^{a}	1115±70^{b}	28.5	41.3	29.7	53.7	74.7	48.7	74.5	83.0
p values	<0.001	0.051	0.003	<0.001								

PA: phytic acid, RV1: red variety 1, RV2: red variety 2, WV1: white variety 1, WV2: white variety 2, SFS: spontaneously fermented slurry, SFP: porridge from SFS, SFP_p: porridge from SFS with peanut butter, BFS: backslopped fermented slurry, BFP: porridge from BFS, BFP_p: porridge from BFS with peanut butter. PA contents are expressed as mg/100 g dm. Values within a column with a different superscript are significantly different ($p < 0.05$), n=2.

Table 3.7: Proximate composition of finger millet sour porridge with and without peanut butter

	SFP				SFP_p				
Parameter	**RV1**	**RV2**	**WV1**	**WV2**	**RV1**	**RV2**	**WV1**	**WV2**	**p values**
Dry matter	10.6±1.1^{a}	11.2±1.2^{a}	10.8±1.2^{a}	11±1^{a}	14.5±0.6^{b}	20.3±1.0^{d}	16.9±2.2^{c}	18.0±0.8^{c}	<0.001
Carbohydrate	9.28±1.05^{a}	9.86±0.39^{a}	9.51±0.78^{a}	9.44±0.34^{a}	9.87±0.51^{a}	12.8±0.77^{a}	12.3±2.62^{a}	10.8±0.48^{a}	0.073
Protein	0.87±0.07^{a}	0.77±0.09^{a}	0.74±0.03^{a}	1.03±0.07^{a}	1.80±0.09^{b}	2.97±0.52^{c}	1.78±0.28^{b}	3.08±0.48^{c}	<0.001
Lipid	0.19±0.01^{a}	0.17±0.01^{a}	0.14±0.01^{a}	0.18±0.03^{a}	2.44±0.09^{b}	3.87±0.18^{c}	2.31±0.35^{b}	3.64±0.34^{c}	<0.001
Ash	0.27±0.03^{a}	0.41±0.07^{b}	0.39±0.01^{b}	0.35±0.02^{a,b}	0.42±0.01^{b,c}	0.64±0.11^{c,d}	0.55±0.04^{d}	0.54±0.05^{d}	<0.001
Energy density[1]	62.4±4.6^{a}	64.1±1.0^{a}	62.2±3.0^{a}	63.5±1.1^{a}	88.6±3.3^{b}	118±1^{d}	97.0±1.1^{b,c}	108±4^{c,d}	<0.001

RV1: red variety 1, RV2: red variety 2, WV1: white variety 1, WV2: white variety 2, SFP: spontaneously fermented porridge, SFP_p: spontaneously fermented porridge with peanut butter. [1]Total energy was calculated including addition of 5 g sugar (20 kcal) for all porridges. Proximate composition is expressed as g/100 g fresh weight while energy density is expressed in kcal/100 g fresh weight. Values across rows with a different superscript are significantly different ($p < 0.05$), n=2.

Table 3.8: Iron and zinc contents of finger millet flour, fermented slurries and porridges

	Iron				Zinc			
	RV1	**RV2**	**WV1**	**WV2**	**RV1**	**RV2**	**WV1**	**WV2**
Flour	1.35±0.12^{a}	0.78±0.02^{a}	1.29±0.08^{a}	2.05±0.06^{a}	0.96±0.19^{a}	0.94±0.08^{a}	1.01±0.03^{a}	1.28±0.03^{a}
SFS	2.87±0.28$^{b.c}$	1.08±0.14$^{a.b}$	2.29±0.07^{b}	2.29±0.01$^{a.b}$	1.16±0.13^{a}	1.25±0.11^{b}	1.44±0.17^{b}	0.99±0.02^{a}
SFP	2.3±0.1^{b}	1.23±0.10$^{a.b.c}$	2.51±0.16^{b}	2.82±0.01^{b}	1.16±0.44^{a}	1.28±0.05^{b}	1.21±0.11^{c}	1.55±0.07^{b}
SFP_p	2.57±0.44^{b}	1.70±0.03^{c}	2.15±0.06^{b}	2.09±0.19^{a}	1.32±0.11^{a}	1.60±0.01^{c}	1.33±0.03$^{b.c}$	1.73±0.51^{b}
BFS	3.22±0.22^{c}	1.27±0.39$^{a.b.c}$	2.08±0.18^{b}	2.71±0.17^{b}	1.31±0.22^{a}	1.41±0.17$^{b.c}$	1.06±0.05^{a}	0.99±0.07^{a}
BFP	2.41±0.08^{b}	1.14±0.06$^{a.b}$	2.37±0.12^{b}	2.57±0.18$^{a.b}$	1.28±0.61^{a}	1.19±0.06$^{a.b}$	1.05±0.09^{a}	1.60±0.11^{b}
BFP_p	2.76±0.12$^{b.c}$	1.58±0.06$^{b.c}$	2.62±0.14^{b}	1.78±0.46^{a}	1.23±0.05^{a}	1.56±0.08^{c}	1.16±0.04$^{a.c}$	1.33±0.09$^{a.b}$
P values	<0.001	<0.001	<0.001	0.013	0.270	<0.001	<0.001	0.002

RV1: red variety 1, RV2: red variety 2, WV1: white variety 1, WV2: white variety 2, SFS: spontaneously fermented slurry, SFP: porridge from SFS, SFP_p: porridge from SFS with peanut butter, BFS: backslopped fermented slurry, BFP: porridge from BFS, BFP_p: porridge from BFS with peanut butter, dm: dry matter. Iron and zinc content is expressed as mg/100 g dm. Values within a column with a different superscript are significantly different ($p < 0.05$), n=2.

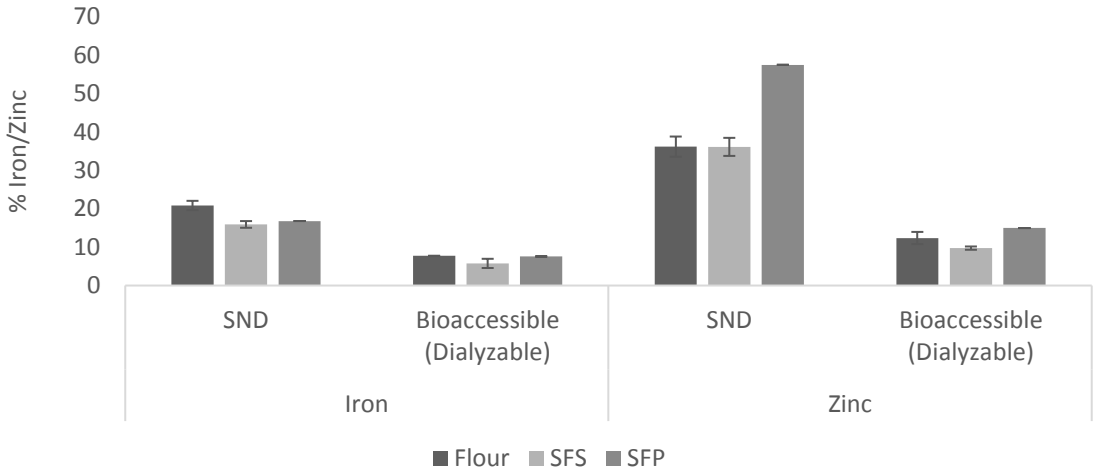

Figure 3.3: Iron and zinc bioaccessibility of finger millet flour, fermented slurry and porridge of red variety 2

RV2: red variety 2, SND: soluble non dialyzable fraction, SFS: spontaneously fermented slurry, SFP: porridge from SFS, n=2.

3.5 Discussion 1: Effect of household fermentation and cooking on soluble and bound phenolic compounds

The PC in finger millet are known to be mainly concentrated in the seed coat of the grain (Chethan and Malleshi, 2007) and are present both in soluble and bound form and also occurring more abundantly in the red varieties than in the white varieties. A major proportion of PC found in cereal grains are in the bound form and can be up to 91 % for some maize varieties (Acosta-Estrada et al., 2014; Adom and Liu, 2002). The bound PC are mainly phenolic acids and CT bound to lignin, hemicellulose, structural proteins and carbohydrates through ether and ester linkages (Acosta-Estrada et al., 2014). In the present study, approximately 95 % of the total PC were in the bound form and, to our knowledge, such associations have never been reported in finger millet (Figure 3.1 and 3.2). A high amount of phenolic acids in both red and white varieties was also found in the bound fraction (Table 3.4 and 3.5). The chief hydroxycinnamic acids, ferulic, *p*-coumaric, caffeic and sinapic acid are known to be ester linked to various cell wall polymers such as arabinoxylans thus they exist in high amounts in the bound fraction (N'Dri et al., 2013; Subba Rao and Muralikrishna, 2002). Ferulic acid was reported as the major bound phenolic acid in cereal grains (Adom and Liu, 2002; Mattila et al., 2005; Subba Rao and Muralikrishna, 2001, 2002) and was also in the present study the most abundant phenolic acid in the bound fraction of both the white and red varieties. Subba Rao and Muralikrishna reported ferulic acid content of 186 µg/g in finger millet and this is in agreement with our findings of 107-193 µg/g. The phenolic acids

identified in the present study have also been reported by others (Chandrasekara and Shahidi, 2010, 2011; Dykes and Rooney, 2006) but their amounts varied largely showing that the occurrence of different PC in grains may depend on several factors such as environmental, edaphic agricultural and varietal differences.

Fermentation caused an increase in the soluble PC, CT and individual PC and a decrease in the bound ones (Table 3.2 and 3.3). Different mechanisms have been suggested to explain the changes occurring to PC and CT after fermentation. The low pH attained during fermentation can cause the removal of hydride ions and reorganization of PC (Towo et al., 2006), some PC may self-polymerize and also interact with other macromolecules such as proteins thus decreasing their extraction, polymerized PC may be degraded into oligomeric PC thus increasing the content of the soluble PC (Duodu, 2014; Taylor and Duodu, 2015). Also the synthesis or enzymatic transformations of PC may occur during fermentation (Taylor and Duodu, 2015; Wang et al., 2014). During fermentation, enzymes from both the cereal matrix and microbes may result in structural breakdown of cell walls thereby increasing the accessibility of bound PC to enzymatic attack. Tannin-protein and tannin-iron complexes are hydrolyzed during fermentation therefore the reduction of PC and CT after fermentation could improve the digestibility of proteins and the bioaccessibility of trace minerals such as iron and zinc (Onweluzo and Nwabugwu, 2009). It also implies that a controlled fermentation with the use of starter cultures suitable for degradation of PC and CT could result in improved protein and mineral nutrition. The increase of phenolic acids in the soluble fraction as observed in table 3.4 and 3.5 suggest that esterase and glucosidase activities may have been rampant during fermentation as their activities are known to release phenolic acids bound to cell walls in particular protocatechuic and p-hydroxybenzoic acids (Svensson et al., 2010).

The effect of thermal processes on PC is dependent on the type of thermal process employed, i.e. wet cooking, steam cooking, extrusion cooking, baking and roasting (Hithamani and Srinivasan, 2014). In our case, wet cooking was employed during the preparation of porridge. The further increase of soluble PC, soluble CT and individual soluble PC after cooking was consistent in all millet samples as shown in Table 3.2-3.3. In contrast to soluble PC, there was a variable response to bound PC and CT after cooking. According to Duodu (2014), PC in grains can increase, decrease or not change after processing depending with the mechanism of action of the processing method employed. The decrease of bound PC, CT and phenolic acids could be a result of leaching of PC into the cooking water with subsequent increase of the soluble PC. Increases of soluble PC observed in this study are caused by processes that release bound PC making them more extractable and assayable. Such processes may involve depolymerization reactions. The increase of the soluble PC and CT in our study could be mainly

attributed to the increase of catechin, particularly in the red varieties. This is because finger millet contains proanthocyanidins which are largely polymers of catechin which were depolymerized during fermentation and cooking. The catechin data (Table 3.4 and 3.5) shows that it is the main flavonoid occurring in the soluble fraction. Catechin accounts for 84% of the soluble fraction in finger millet (Chandrasekara and Shahidi, 2011). The vanillin method used to measure CT is a crude method that unfortunately, also measures catechin, explaining the observed increase of CT in the soluble fraction. The CT in finger millet have not been characterized in detail like CT in sorghum but they are likely to be oligomers of catechin as catechin, epicatechin, gallocatechin, epigallocatechin, trimers and tetramers of catechin and procyanidin dimers B1 and B3 have been identified in finger millet (Chandrasekara and Shahidi, 2011; Shobana et al., 2009). The content of bound PC increased in the red varieties but decreased in the white varieties after cooking. The decrease of bound PC and phenolic acids in WV1 (Table 3.5) may be as a result of the cooking process employed. After fermentation, the slurry was decanted and the coarse particles containing the seed coat with the highest PC was discarded. The red varieties of finger millet contain CT whose extractability could have been increased after thermal treatment. Several authors have found a decrease of total PC after cooking of sorghum, fonio, pearl, kodo, finger, foxtail, proso and little millet (Chandrasekara et al., 2012; Hithamani and Srinivasan, 2014; N'Dri et al., 2013; Towo et al., 2003) while others reported increases (Pradeep and Guha, 2011). Varying effects of thermal treatment on bound PC and CT of different type of grains have also been reported by other workers (Hithamani and Srinivasan, 2014; Svensson et al., 2010; Wu et al., 2013).

Soluble PC are stored in the intracellular space of cells while bound PC are located in proximity and in association with cell walls (Yeo and Shahidi, 2015). The increase of soluble PC coupled with general decrease of bound PC after fermentation and cooking alludes to the fact that there is depolymerisation of bound PC and their transportation into the intracellular space. As the decrease of the bound PC did not add up with the increase of the soluble PC, it suggests that some other chemical and physical interactions come into play. The changes occurring to soluble and bound phenolics can thus allow for prediction of localization, transport mechanism and chemistry of phenolics during processing (Yeo and Shahidi, 2015).

Several studies have shown improved mineral bioaccessibility after reduction of PC and CT (Lestienne et al., 2005a; Towo et al., 2006). The reduction of CT in cereals is encouraged as it leads to products with reduced astringency, protein digestibility and mineral bioavailability (Dlamini et al., 2007). On the other hand, fermentation and/or cooking has also been shown to effect changes on PC and CT which subsequently increase the antioxidant capacity of the products (Gan et al., 2016; Kayodé et al., 2013;

Pradeep and Guha, 2011). Kumari et al (Kumari et al., 2016) revealed that even though bound PC were higher than soluble PC in finger millet, the antioxidant activity of the soluble extract measured by various antioxidant tests i.e. DPPH radical scavenging activity, trolox equivalent antioxidant activity, β-carotene-linoleate model system and ferrous ion-chelating activity, was consistently at least twice that of the bound extracts. Yeo and Shahidi (Yeo and Shahidi, 2015) proposed to use the ratio of bound to soluble PC as an estimate to predict the localization, transportation mechanisms and antioxidant activity of phenolics in germinating lentils. The ratio of bound to soluble PC of our samples decreased from 16.7-20.8 in the flour to 2.18-5.67 after fermentation and cooking. Using this ratio of bound to soluble PC, we can postulate that the antioxidant activity after fermentation and cooking will increase by a large magnitude. However, the benefits of finger millet phenolics go beyond their antioxidant activity. Future research should thus focus on determining whether the increase in soluble PC also improves the absorption of PC via *in vivo* and *in vitro* studies. Increased absorption is a prerequisite for the phenolics to demonstrate their purpoted health effects significantly. In addition, since PC are also implicated in the inhibition of mineral absorption, it is also important to determine if an association exists between PC and mineral bioavailability.

3.6 Discussion 2: Effect of household fermentation and cooking on proximate composition, mineral binders and subsequent iron and zinc bioaccessibility

Cereal based porridges are normally the first solid foods given to infants in Zimbabwe to supplement breastmilk. Their nutritional adequacy is of great relevance for the prevention of malnutrition and micronutrient deficiencies in infants and children. Porridges with just water and cereal flour had a low dry matter content (< 10%), while by adding peanut butter the dry matter content increased to 17.5% (Table 3.7). A dry matter content of less than 10% is normally not sufficient to meet energy and nutrient requirements. According to Dewey and Brown (2003), energy intake from complementary foods should be 84 kcal/100 g assuming a consumption of 2 meals/day and average breastmilk intake for children aged between 9-11 months. The SFP and BFP thus fails to meet the energy requirement. Low dry matter and energy density of 7% and 41 kcal/100 g respectively has been observed for *bensaalga*, a traditionally fermented pearl millet gruel (Mouquet-Rivier et al., 2008). Low values of < 12% dm have also been reported for *togwa* (Ndabikunze et al., 2001). Thirty millet porridges sampled from Cote D'Ivoire had higher dry matter content of 13-24% with an average of 77 kcal/100 g energy which still is not sufficient to meet energy requirements for children below the age of one. As cereals do not contain substantial amounts of protein and lipids, the fortification of porridges with other high fat or protein foods such as legumes is beneficial and has been found to be successful in terms of improving

the macronutrient balance. Examples of legumes used include groundnuts used as peanut butter in this study, soybeans and cowpeas (Oluwamukomi et al., 2005; Tou et al., 2007a). Indeed, adding peanut butter increased the energy value in this study by 42-87%.

The bioavailability of minerals is influenced by the presence of mineral binders such as PA, PC and CT. Fermentation is known to reduce PA as a result of the low pH environment created causing the activation of endogenous phytases and the production of microbial phytases (Coulibaly et al., 2011). The PA (IP6) is broken down into lower inositol phosphates which are less potent in mineral binding. Millets have a low endogenous phytase activity (Brinch-Pedersen et al., 2014), thus a reduction of PA during fermentation largely depends on the production of microbial phytases. A reduction of PA of more than 50% after spontaneous fermentation has been observed during the preparation of sorghum and millet African porridges under standardized conditions (Kayodé et al., 2013; Kruger et al., 2012; Mouquet-Rivier et al., 2008; Proietti et al., 2013; Tou et al., 2007a; Towo et al., 2006). The general lack of reduction of PA after fermentation (Table 3.6) was thus unforeseen and since PA reduction was only observed in one variety, it can be inferred that traditional fermentations are highly unpredictable because of differences that arise from many parameters influencing the fermentation. The reduction of PA after fermentation is also aided by pre-processing steps such as decortication, soaking and germination (Kayodé et al., 2013; Kayodé et al., 2006; Makokha et al., 2002; Traoré et al., 2004) processes which are not employed during the preparation of Zimbabwean sour porridge because of cultural differences.

There are conflicting findings concerning the effect of cooking or boiling on PA as some authors reported no change in PA after cooking (Kayodé et al., 2007b), a decrease in PA of 23-55% after cooking of sorghum (Kayodé et al., 2007a; Towo et al., 2006) while others reported an increase of up to 68% after cooking of previously soaked soybean flour (Lestienne et al., 2005c). Proietti et al. (2013) also reported variable changes to PA after cooking different varieties of sorghum and these changes appear to be influenced by variety and also preprocessing conditions such as fermentation. The increase in PA after cooking as observed in the present study (Table 3.6) could be attributed to an increased extraction of IP6 and IP5 molecules (Lestienne et al., 2005c). High molar ratios were observed for PA/Fe and PA/Zn owing to the high PA content and low mineral levels. The decrease in PA/mineral ratios was not caused by a reduction of PA during processing but was the result of an increase in minerals during processing. For improved bioavailability, it is recommended that the PA/Fe ratio should be < 1 or better still < 0.4 for plant based diets with no enhancers of iron (Hurrell and Egli, 2010). Molar ratios of < 5, 5-18 and > 18, respectively, indicate a high, moderate and low bioavailability for zinc (Hotz and Brown, 2004). The PA/Fe and PA/Zn molar ratios fall way beyond the recommended ratios suggesting remarkably low iron and zinc bioavailability of the porridges as was also observed in the *in vitro* iron

and zinc bioaccessibility. Some changes were also observed in the soluble and bound PC and CT of the finger millet products after fermentation and cooking as discussed in section 3.5.

In terms of the mineral contents, the iron and zinc content of the finger millet was below reported ranges (Table 3.8). An analysis of the core collection of world's finger millet germplasm resulted in iron contents of 2.17–6.52 mg/100 g dm and 1.66-2.53 mg/100 g dm for zinc (Upadhyaya et al., 2011). The low iron content in the finger millet grains could be the result of a low nutrient fertility in the Hwedza soils. According to a report on the physical resource inventory of the communal lands of Zimbabwe, soils from Hwedza communal area are deep weathered, non-fertile sandy loam soils with low free iron content as compared to other areas with at least ten times more free iron (Anderson et al., 1993). In general, we observed an increase in the iron and zinc after processing compared to the flour samples due to their supply from water and cooking utensils, which is inevitable during household processing. The fermentation process was carried out by the addition of variable amounts of water to the flour in plastic or metal containers while the cooking process was done by subjecting the fermented slurries to a heating process after addition of extra water in metal pots. The increase in iron and zinc contents observed is variable as different amounts of water were added, as well as due to the source of water used by the different households. Finger millet is generally considered a good source of iron and zinc with the potential to alleviate mineral deficiencies through increased consumption (Devi et al., 2011). However, the low iron and zinc contents observed in our millet samples showed that mineral contents of cereal grains may not be generalized and may highly depend on environmental conditions and agronomic management. The low iron and zinc content may explain why there are intermittent mineral deficiencies in some areas (Central Statistics Office, 2011).

No significant change in mineral bioaccessibility (measured by the proportion of iron able to pass through a dialysis membrane of 12-14 kDa) was observed after fermentation and cooking (Figure 3.3). The average *in vitro* iron and zinc bioaccessibility was 6.5% and 11%, respectively. Iron bioaccessibility values of 0.3-4% have been reported by several authors after preparation of African fermented cereals (Baye et al., 2015; Baye et al., 2014; Baye et al., 2013; Greffeuille et al., 2011) while values of 5.6-7% (as solubility) have been reported for zinc (Kayodé et al., 2006). The lack of improvement in mineral bioaccessibility after processing could be because PA as IP6 was not degraded to lower forms of inositol phosphate after processing. According to Hurrell and Egli (2010), degradation of more than 90% IP6 is required to envisage a two fold increase in iron bioavailability. Furthermore, Sandberg and Svanberg (1991) recommended that residual IP6 should not be more than 33 mg/100 g to eradicate any inhibitory effect on iron availability. Also, as the PA/mineral ratios are much higher and not in the vicinity of ratios recommended for improved mineral bioavailability, no improvement in iron and zinc bioaccessibility can be expected. Although some reduction of PC and CT was observed, this reduction

was not sufficient to cause an increase in mineral bioaccessibility. On the contrary Baye et al. (2015), found no improvement in iron bioaccessibility even after more than 90% PA reduction in *tef* based food products. These controversies indicate that factors other than PA may decrease the bioaccessibility of Fe and Zn. Recent evidence has shown that to obtain an appreciable improvement in iron and zinc bioaccessibility in cereal grains containing high levels of CT, both PA and CT should be adequately reduced as the mineral binders are intricately associated with each other in the plant matrix (Baye et al., 2015). This therefore suggests that the reductions of mineral binders in our study was not adequate to effect any changes in mineral bioaccessibility.

Given the mineral contents of the porridges and considering 6 and 13% iron and zinc bioaccessibility, respectively, the present finger millet porridges consumed in the Ushe communal area of Zimbabwe cannot meet more than 50% of the total iron and zinc requirement, even at maximum consumption rate (20% dm porridge, 100 g porridge, 3 times /day) whereas complementary porridges should be able to meet 75-100% of recommended mineral intake (Gibson et al., 1998). The recommended dietary allowance (RDA) of iron for children aged between 1-3 years is 5.8 mg/day (assuming 10% bioavailability) while it is 8.3 mg/day (assuming 15% bioavailability) for zinc (World Health Organization, 2004). This finding may help to explain why there is high mineral deficiency and high prevalence of stunting in this area (Central Statistics Office, 2011). As this study was done on actual products consumed by people from Ushe communal area, it offers some real insights on what should be prioritized in terms of intervention for improved mineral nutrition. As some metabolism of all mineral binders was observed in this study, it means that fermentation still has potential to change the trajectory of mineral bioavailability in complementary foods from developing countries. Standardization followed by optimization of traditional fermentations is important to ensure predictable and successful fermented products. Crucially, fermentation process should be optimized to favor the growth of microbes with the ability to adequately metabolize PA, PC and CT. However, the question still remains of what is adequate? To what extent should PC and CT be reduced to have significant improvements in mineral bioaccessibility?

3.7 Conclusion

Fermentation and cooking caused an increase in the soluble PC and CT counteracted by a decrease in the bound PC and CT. Overall, there was a marginal reduction of total PC and CT after fermentation and cooking. On the contrary, PA was only reduced in one variety after fermentation and cooking. The nutritional adequacy of porridges is important as they are dietary staples for children. The energy content of finger millet based sour porridge could only meet recommended levels after addition of

peanut butter. There was no improvement in iron and zinc bioaccessibility due to fermentation and cooking. Furthermore, the iron and zinc contents of the finger millet grains were low such that an improvement in their bioaccessibility will still not be enough to improve mineral nutrition of people in Ushe communal area. Populations subsisting on cereal grains are at risk of suffering from mineral deficiencies such that strategies to improve mineral nutrition are urgently needed. Such strategies may entail increasing grain mineral contents and improved agronomic management, reducing mineral binders through optimized processing methods. Crucially, selection of varieties with higher mineral content and lower PA content should be the major thrust in biofortification initiatives. As these porridges are produced in small scale household conditions and generally consumed by resource poor population groups in developing countries, it is imperative that strategies for their improvement be realistic in terms of ease of production, cultural acceptability and affordability.

Chapter 4

Iron and zinc bioaccessibility of fermented maize, sorghum and millets from five locations in Zimbabwe

Chapter 4 : Iron and zinc bioaccessibility of fermented maize, sorghum and millets from five locations in Zimbabwe

4.1 Abstract

The present study is an evaluation of iron and zinc bioaccessibility of fermented maize, sorghum, pearl millet and finger millet from five different locations in Zimbabwe. Bioaccessibility was defined as the proportion of minerals able to pass through a dialysis membrane of 12-14 kDa molecular weight cut-off. Iron and zinc contents ranged between 3.22-49.7 and 1.25-4.39 mg/100 g dm, respectively. Fermentation caused a reduction of between 20-88% of PA while a general increase in soluble PC and a decrease of the bound PC was observed. Bioaccessibility of iron and zinc ranged between 2.77-26.1% and 0.45-12.8%, respectively. The potential contribution of the fermented cereals towards iron and zinc absolute requirements ranged between 25-411% and 0.5-23% with higher contribution of iron coming from cereals that were contaminated with soil iron. Populations subsisting on cereals could be more at risk of zinc rather than iron deficiency.

Keywords: iron, zinc, bioaccessibility, cereals, phytic acid, phenolic compounds

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4.2 Introduction

Iron and zinc deficiencies are a major public health concern particularly in developing countries where diets are based on monotonous cereal staple foods. Cereal grains such as maize, sorghum and millets commonly consumed in Africa have a high amount of mineral binders such as phytic acid (PA) and phenolic compounds (PC), including condensed tannins (CT) (Gabaza et al., 2017a). These cereal grains are very important for infants and young children as complementary porridges are based on several variations of the fermented cereals. The complementary porridges are normally administered to children 2-3 times a day (Gabaza et al., 2017b; Gibson et al., 1998). In Africa, cereal grains can contribute up to 57 and 60% of total iron and zinc intake, respectively (Joy et al., 2014).

Mineral bioavailability may be dependent on location, growing conditions and variety as there exists a large variation in the mineral and inhibitor contents of the same cereal grain. An assessment of the finger millet, sorghum and pearl millet germplasms showed iron contents of 21-65, 26-61 and 30-78 mg/kg and zinc contents of 17-25, 21-57 and 25-65 mg/kg, respectively (Ashok et al., 2012; Upadhyaya et al., 2011; Velu et al., 2007). On the other hand, a large diversity in the content of PA, PC and CT has also been reported for sorghum and millets (Dykes and Rooney, 2006; Kumari et al., 2017; Ravindran et al., 1994). These differences in mineral and mineral binder contents suggest also a large diversity in mineral bioavailability.

Many complementary porridges in Africa are based on spontaneously fermented cereals because of their impact on nutrition, health and socioeconomy of the people, as such fermentation process is of interest (Franz et al., 2014). Sour porridge is one such type of fermented porridge consumed in Zimbabwe and is prepared using maize, finger millet, pearl millet or sorghum. It is prepared by spontaneously fermenting a cereal flour: water mixture for 24-36 hours at ambient temperature, to attain a pH of around 4.0 followed by a cooking process to produce the porridge (Gabaza et al., 2016). The lactic acid bacteria (LAB) associated with these fermented cereals show variation based on type and variety of cereal, fermentation conditions, duration of fermentation etc. hence their action on mineral binders also widely varies (Gabaza et al., 2017a). The bioavailability of iron and zinc in fermented cereals is thus dependent on the interaction of many factors.

The bioavailability of iron in fermented cereals can also be influenced by the presence of extrinsic iron which can become part of the food due to contamination from soil, dust, processing equipment and preparation procedures (Greffeuille et al., 2011). In particular, cereal grains that are associated with traditional threshing practices such as sorghum and millets may be highly contaminated with iron from soil. A study by Gibson et al. (2015) revealed that women from Zombwe in Malawi had high total body iron despite their consumption of a diet with high mineral binders. This was attributed to

contamination of millet with acidic soils during the threshing process. Henceforth, contaminant iron, if bioavailable, maybe an important part of the diet in some areas (Gibson et al., 2015).

Therefore, the objective of the present study was first, to determine the effect of fermentation on mineral binders in cereals from different locations and secondly, to gain an understanding of their subsequent iron and zinc bioaccessibility (proportion of iron and zinc likely to be absorbed after release from food matrix due to digestion). The iron and zinc contents and bioaccessibility information will be used to estimate the relative contribution of cereal porridges to the iron and zinc absolute requirements (amount of required bioavailable iron and zinc) of children aged between 1-3 years. This study may provide insight on the vulnerability of certain locations to both iron and zinc deficiencies as well as variation in mineral bioaccessibility of different cereal grains.

4.3 Materials and methods

4.3.1 Materials

Cereal grains and water were obtained in communal areas from five different locations in Zimbabwe, namely Chiweshe, Chiredzi, Mutoko, Hwedza and Victoria Falls **(Figure 4.1).** Folin Ciocalteu's reagent, gallic acid, vanillin, catechin, α-amylase from porcine pancreas (Type VI-B, > 10 units/mg solid), pepsin from porcine gastric mucosa lyophilized powder (3200-4500 units/mg protein), pancreatin from porcine pancreas (8xUSP), dialysis bags (MW cut off 12-14 kDa), bile from porcine bile extract, phytic acid dodecasodium salt, 2,2' bipyridine and thioglycolic acid were all Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA) products. ICP multi-element standard solution IV was procured from Merck (Germany).

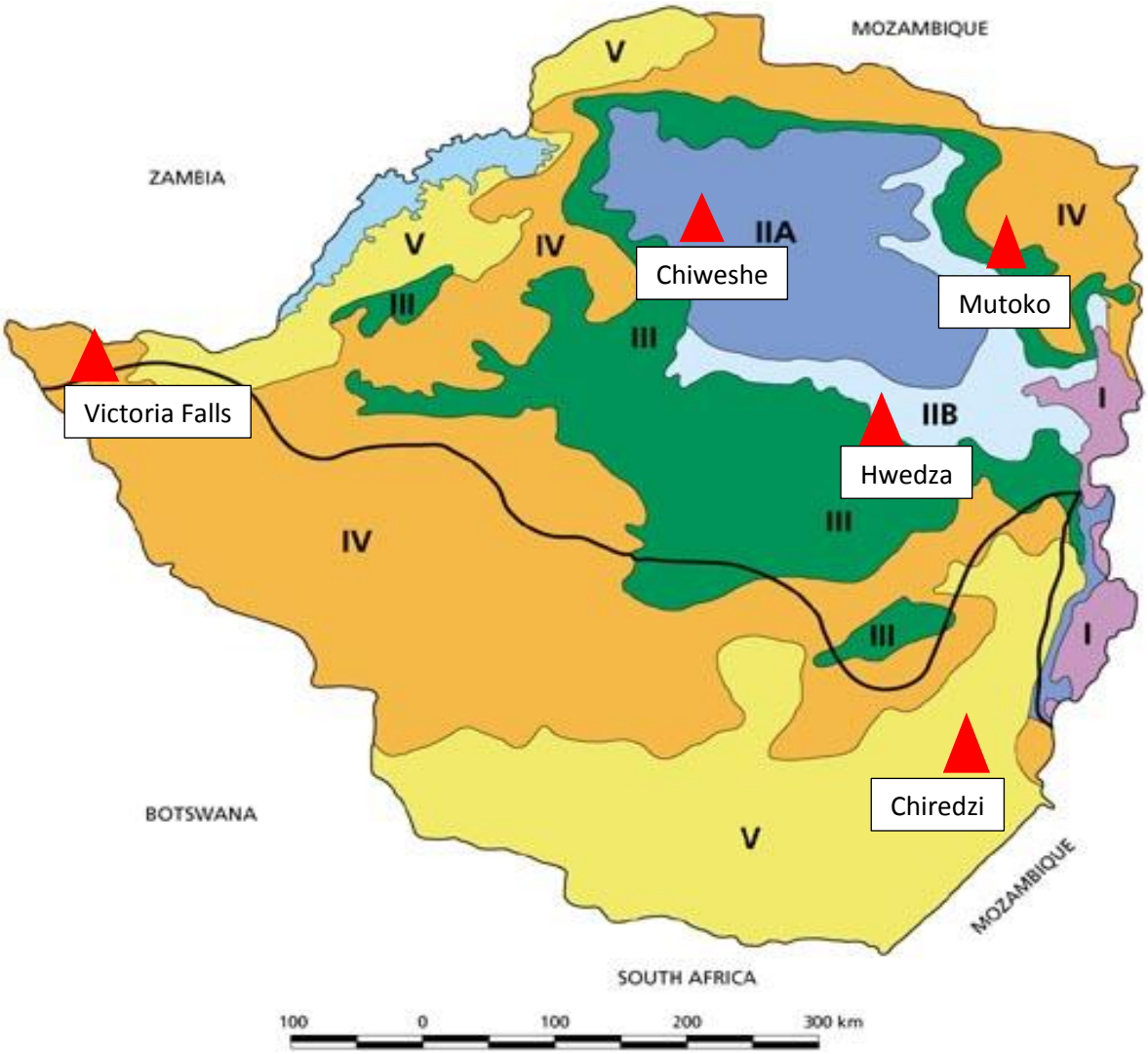

Figure 4.1: Map of Zimbabwe showing agro-ecological zones and the five locations where samples were collected

Points marked with red triangles show the five locations where raw materials to prepare fermented slurries were collected from. I: Specialized and diversified farming region (rainfall > 1000mm), IIA: Intensive farming region (rainfall 750-1000 mm), IIB: Intensive farming region (rainfall 750-1000 mm), III: Semi-intensive farming region (rainfall-650-800 mm), IV: Semi-extensive farming region (rainfall-450-650 mm), V: Extensive farming region (rainfall <650 mm).

4.3.2 Methods

4.3.2.1 Collection of raw materials

Raw materials were collected from five locations in Zimbabwe from the period July 10 to August 12, 2016. Locations (**Figure 4.1**) were chosen based on the availability of most or all of the cereal grain types. At least 200 g of each cereal grain (maize, sorghum (red and white), pearl millet and finger millet (red and white)) was collected from 5-7 households per location. Cereal grains were collected from households with more or less the same agricultural practices based on the information supplied by the agricultural extension workers in these areas. The maize, white sorghum from all the locations and pearl millet from Victoria falls were commercial varieties while the rest of the pearl millet, finger millet and red sorghum were landraces. For each location, a water sample (1 L) was also collected from the most utilized water source in the area. **Table 4.1** shows the type of samples that were collected at each location.

4.3.2.2 Preparation of fermented slurries

Both cereal and water samples were transported to the laboratory under cooled conditions at the University of Zimbabwe where the water samples were immediately stored at 4 ^{o}C prior to use. The cereal grains were further dried overnight in an oven at 60 ^{o}C, to ensure dryness and then carefully cleaned to remove glumes and foreign particles. For each location and cereal grain type, a composite sample was made by mixing equal aliquots of the cereal grain from the different households in that location in order to produce a sample representative of each location. A total of 18 composite samples were prepared according to the number of different cereal types collected per location (**Table 4.1**). Composite samples were milled to make flour using a laboratory hammer mill equipped with sieve of size 0.5 mm. To determine possibility of iron contamination during milling, some cereal grains were powdered using pestle and mortar and their iron contents were compared with the hammer milled cereal grains. No difference was observed as such all cereal grains were milled using the laboratory hammer mill. Fermented slurries were prepared using the specific water that is used for drinking and cooking purposes at the same location where the cereal samples came from without further treatment of the water as this is the normal practice in all the locations. An aliquot of cereal flour (dry milled grain) and water was mixed in the ratio 1:3 in sterile glass jars and the mixture was left to spontaneously ferment for 26 h at 25 ^{o}C as described in section 3.3.2. The pH of the slurries at the end of the fermentation ranged between 4.1-5.7. All fermentations were done in triplicate. At the end of the fermentation, the fermented slurries were transferred to 50 mL sterile falcons and stored at -20 ^{o}C until they were freighted under dry ice together with flour samples to the Laboratory of Food

Microbiology and Biotechnology, Ghent University, Belgium during the period September 14-19, 2016. Thereafter, samples were stored at -20 ^{o}C until further analysis.

Table 4.1: Locations where raw materials for the preparation of fermented slurries were collected from.

Location	Cereals collected	Water source
Chiweshe	maize	borehole
	finger millet	
	sorghum*	
Chiredzi	maize	open well
	finger millet	
	sorghum	
	pearl millet	
Mutoko	maize	borehole
	finger millet	
	sorghum	
	pearl millet	
Hwedza	maize	borehole
	finger millet[§]	
	sorghum*	
	pearl millet	
Victoria Falls	finger millet	borehole
	sorghum	
	pearl millet	

*Sorghum type is red variety while the rest are white varieties. [§] Finger millet type is white variety while the rest are red varieties.

4.3.2.3 Analysis

Determination of dry matter, iron and zinc contents, mineral binders (PA, PC and CT) and *in vitro* iron and zinc bioaccessibility was performed as described in section 3.3.2. Iron and zinc contents of the water from each location was also determined. Iron contents of cereal flours and fermented slurries from Chiweshe and Chiredzi were unusually high and contamination with extrinsic iron was suspected. To determine if this contamination was emanating from soil, aluminum contents of cereal flours and

fermented slurries were also determined. Bioaccessibility was defined as the proportion of minerals able to through a dialysis membrane of 12-14 kDa molecular weight cut-off.

The contribution that the porridges can make towards the absolute requirements and recommended dietary allowances (RDA) of iron and zinc were also calculated using the following criterion: absolute requirement for children between the ages of 1-3 years is 0.58 (95th percentile) and 1.2 mg/day respectively, RDA for iron and zinc for the same age group is 5.8 mg/day (assuming 10% bioavailability) and 8.3 mg/day (assuming 15% bioavailability), respectively. The contribution of iron and zinc in fermented slurries towards the absolute requirement was calculated using % bioaccessibility (assuming that bioaccessibility measured in this study is a good estimation of bioavailability) while the total mineral contents were used to calculate the contribution towards the RDA. A maximum consumption of porridge at 20% dry matter, 100 g portion, 3 times a day was considered which is also equivalent to 20% dry matter, 150 g portion, 2 times a day and this was estimated based on the information gathered in chapter 2.

4.3.2.4 Statistical Analysis

Data were subjected to two-way ANOVA and in all cases, there was interaction between the main effects of location and cereal grain type thus simple effects were considered. Where differences existed, comparisons of means was done using Tukey's post-hoc test ($p < 0.05$). Pearson´s correlation was performed to determine the correlation between iron and aluminum in fermented cereal slurries. All analysis were carried out using IBM SPSS software version 23. Data is presented in tables as means of triplicate independent samples.

4.4 Results

4.4.1 Total phenolic compounds and condensed tannins of flours and fermented slurries

Table 4.2 and 4.3 show the results of the total PC and CT in flour and fermented slurries from different locations in Zimbabwe. Total PC values were the sum of soluble and bound PC and ranged from 468-2208 mg GAE/100 g dm for flour samples and 399-2489 mg GAE/100 g dm for fermented slurries. Lower levels of total PC of <1000 mg GAE /100 g dm were observed for maize, white sorghum, pearl millet and white finger millet while higher levels of >1000 mg GAE/100 g dm were recorded for red finger millet and red sorghum. The largest contribution of soluble PC to total PC was observed for red sorghum (32-36%), followed by pearl millet (8.85-27.7%), maize (7.73-8.65%), white sorghum (3.94-8.21%) and red and white finger millet (4.08-6.86%). The total PC generally increased after

fermentation although in the case of sorghum from Mutoko, there was a slight decrease and no change was observed for the white finger millet from Hwedza. The changes occurring to the total PC can be clearly observed by variations in the bound/soluble ratio. The bound/soluble ratio generally decreased for maize (11.9 to 6.70), white sorghum (12.2 to 6.92), pearl millet (7.65 to 2.76), white finger millet (14.6 to 8.16), red finger millet (20.4 to 14.5) while it increased for red sorghum (1.77 to 2.31), pearl millet (6.78 to 10.3) and finger millet from Victoria Falls (23.6 to 28.4). The changes in the bound/soluble ratio infer an increase in the soluble fraction counteracted by a decrease in the bound fraction for all cereal grains except red sorghum and the samples from Victoria Falls.

Condensed tannins were detected in red sorghum and red finger millet (limit of detection for CT – 0.008 mg/L) (**Table 4.3**). Also in pearl millet, CT were observed, although in a very low concentration (between 4.5-6 mg CE/100 g) and no change due to fermentation in CT was measured so this was considered as background measurement. CT in red sorghum ranged from 515-524 mg CE/100 g dm while in red finger millet they ranged from 44.8-197 mg/100 g dm. After fermentation, CT in both red sorghum and red finger millet increased by up to 83% and 114% respectively.

4.4.2 Phytic acid contents of flours and fermented slurries

The PA content in the flour and fermented slurries (**Table 4.4**). Maize had the highest PA content from all locations except Chiredzi. Fermentation caused a variable decrease in PA for all cereal types. Reduction of PA ranged from 20-88% with the highest reduction observed for white sorghum from Victoria Falls and the lowest reduction for maize from Chiredzi. Even though there was a high reduction of PA in some samples, the residual PA in all the samples was still high and ranged between 157-1166 mg/100 g dm. The PA/Fe ratios of the fermented slurries were reduced from 2.68-34.8 to 0.274-15.8 while for zinc, PA/Zn was reduced from 12.4-97 to 4.04-51.9.

4.4.3 Iron and zinc contents of flours and fermented slurries

The iron and zinc contents of the water from all locations was negligible (< 0.01 mg/L) as such no difference was observed between the iron and zinc contents of the cereal flours and the fermented slurries. Iron and zinc contents in fermented slurries are shown in **Table 4.5**. The total mineral contents were a result of the interaction of each cereal with its environmental conditions ($p < 0.05$). Iron contents in all fermented slurries ranged between 3.22-49.7 mg/100 g dm with the highest levels recorded for pearl millet (9.13-49.7 mg/100 g dm) at all different locations. Higher iron contents were observed in all cereals from two locations, namely Chiweshe and Chiredzi. Zinc contents ranged between 1.25-4.39 mg/100 g dm with pearl millet again having the highest zinc contents averaging

4.33 mg/100 g dm. Finger millet had consistently lower levels of zinc content with an average of 1.79 mg/100 g dm.

Table 4.2: Total phenolic contents of cereal flours and fermented slurries from different locations in Zimbabwe

	Chiweshe	Chiredzi	Mutoko	Hwedza	Victoria Falls	SEM	P value
				Flour			
Maize	535^{a,A}	541^{a,b,B,C}	590^{a,b,B,C}	631^{a,b,C}	ns	20.6	0.034
Sorghum	2154^{c,C}*	469^{a,A}	459^{a,A}	2208^{c,C}*	1052^{b,B}	42.7	< 0.001
P/millet	ns	599^{b,A}	607^{b,B,C}	675^{b,B,C}	705^{a,C}	23.2	0.031
F/millet	1471^{b,D}	1077^{c,B}	1253^{c,C}	468^{a,A§}	1342^{c,C,D}	32.3	< 0.001
SEM	42.5	20.1	30.2	39.5	20.6		
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		
				Fermented slurries			
Maize	651^{a,B,C}	597^{a,A}	624^{b,B,C}	705^{b,C}	ns	22.4	0.046
Sorghum	2489^{c,E}*	537^{a,B}	399^{a,A}	2082^{d,D}*	813^{a,C}	23.7	< 0.001
P/millet	ns	629^{a,A}	618^{b,A}	820^{c,C}	704^{a,B,C}	29.0	< 0.001
F/millet	1488^{b,B}	1320^{b,B}	1585^{c,B}	468^{a,A§}	1302^{b,B}	79.4	0.004
SEM	90.7	38.4	33.0	12.3	37.5		
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		

*Sorghum type is red variety while the rest are white varieties. § Finger millet type is white variety while the rest are red varieties. P/millet: pearl millet, f/millet: finger millet, SEM: standard error of means. Values are expressed as mg GAE/100 g dm. Values with different small superscript letters within columns are significantly different. Values with different capital superscript letters across rows are significantly different, $p < 0.05$, n=3, ns – no sample, GAE – gallic acid equivalents.

Table 4.3: Content of condensed tannins in flours and fermented slurries from different locations in Zimbabwe

	Chiweshe	Chiredzi	Mutoko	Hwedza	Victoria Falls	SEM	P value
				Flour			
Sorghum	515^{b}*	nd	nd	524*	nd	27.5	0.818
F/millet	44.8^{a,A,B}	62.1^{B}	197^{C}	nd	24.3^{A}	4.44	<0.001
SEM	12.7	3.56	7.32	24.4	5.0		
P value	< 0.001	-	-	-	-		
				Fermented slurries			
Sorghum	752^{a,A}*	nd	nd	957^{B}*	nd	24.6	0.004
F/millet	95.9^{b,B}	82.3^{B}	154^{C}	nd	51.8^{A}	3.99	0.001
SEM	22.4	3.02	5.08	10.9	3.27		
P value	< 0.001	-	-	-	-		

*Sorghum type is red variety while the rest are white varieties. § Finger millet type is white variety while the rest are red varieties. f/millet: finger millet, SEM: standard error of means. Values are expressed as mg CE/100 g dm. Values with different small superscript letters within columns are significantly different. Values with different capital superscript letters across rows are significantly different, $p < 0.05$, n=3, nd – not detected (limit of detection – 0.008 mg/L), CE – catechin equivalents.

Table 4.4: Phytic acid contents of cereal flours and fermented slurries from different locations in Zimbabwe

	Chiweshe	Chiredzi	Mutoko	Hwedza	Victoria Falls	SEM	P value
				Flour			
Maize	1998^{c,B}	607^{a,A}	2148^{c,B}	2197^{d,B}	ns	57.9	<0.001
Sorghum	548^{a,A*}	819^{b,A}	1648^{b,B}	703^{a,A*}	1973^{c,B}	71.1	<0.001
P/millet	ns	588^{a,A}	1362^{b,B}	1232^{c,B}	1382^{b,B}	25.8	<0.001
F/millet	919^{b,B}	984^{b,B,C}	757^{a,A}	1043^{b,C,D,§}	1132^{a,D}	59.7	<0.001
SEM	44.5	40.6	91.8	31.2	45.1		
P value	<0.001	<0.001	<0.001	<0.001	<0.001		
				Fermented slurries			
Maize	1166^{b,C}	483^{c,A}	1099^{c,B,C}	819^{b,B}	ns	62.3	0.001
Sorghum	429^{a,A*}	285^{b,A}	845^{b,c,B}	392^{a,A*}	228^{a,A}	50.0	0.001
P/millet	ns	157^{a,A}	703^{a,b,B}	743^{b,B}	662^{b,B}	59.6	0.001
F/millet	566^{a}	478^{c}	419^{a}	661^{b,§}	517^{a,b}	65.9	0.121
SEM	73.0	20.1	63.1	48.7	81.9		
P value	0.001	< 0.001	< 0.001	0.001	0.025		

*Sorghum type is red variety while the rest are white varieties. § Finger millet type is white variety while the rest are red varieties. P/millet: pearl millet, f/millet: finger millet, SEM: standard error of means. Values are expressed as mg/100 g dm. Values with different small superscript letters within columns are significantly different. Values with different capital superscript letters across rows are significantly different, $p < 0.05$, n=3, ns – no sample.

Table 4.5: Iron and zinc contents of cereal fermented slurries from different locations in Zimbabwe

	Chiweshe	Chiredzi	Mutoko	Hwedza	Victoria Falls	SEM	P value
				Fe			
Maize	16.8^{a,B}	38.8^{a,b,C}	5.93^{a,A}	3.22^{a,A}	ns	1.09	< 0.001
Sorghum	25.9^{b,B}*	33.2^{a,C}	4.73^{a,A}	8.09^{b,A}*	5.62^{a,A}	0.799	< 0.001
P/millet	ns	49.7^{b,B}	9.13^{b,A}	13.6^{c,A}	15.7^{b,A}	2.66	< 0.001
F/millet	12.2^{a,B}	38.9^{a,b,C}	4.46^{a,A}	4.76^{a,A§}	5.34^{a,A}	0.663	< 0.001
SEM	1.18	2.77	0.668	0.396	0.617		
P value	0.001	0.180	0.004	< 0.001	<0.001		
				Zn			
Maize	2.26^{A}	2.34^{a,A}	3.42^{b,B}	3.17^{b,B}	ns	0.161	0.001
Sorghum	1.90*	2.23^{a}	1.89^{a}	1.56^{a}*	1.89^{a}	0.242	0.466
P/millet	ns	3.83^{b}	4.30^{b}	4.39^{c}	4.81^{b}	0.384	0.403
F/millet	1.85^{A,B}	2.42^{a,B}	1.55^{a,A}	1.25^{a,A§}	1.86^{a,A,B}	0.146	0.002
SEM	0.146	0.267	0.292	0.217			
P value	0.172	0.009	< 0.001	< 0.001	<0.001		

*Sorghum type is red variety while the rest are white varieties. § Finger millet type is white variety while the rest are red varieties. P/millet: pearl millet, f/millet: finger millet, SEM: standard error of means. Values are expressed as mg/100 g dm. Values with different small superscript letters within columns are significantly different. Values with different capital superscript letters across rows are significantly different, $p < 0.05$, n=3, ns – no sample.

4.4.4 Iron and zinc bioaccessibility

Table 4.6 and 4.7 shows the %SND and bioaccessibility (%) (derived from the proportion of minerals which were able to pass through a dialysis membrane of 12-14 kDa) of iron and zinc. The %SND ranged between 10.6-65.0% and 11.8-53.5% for iron and zinc, respectively. Pertaining to the bioaccessibility (%), it ranged from 2.77-26.1% for iron and 0.45-12.8% for zinc. The absolute values for iron bioaccessibility ranged between 0.25-3.78 mg/100 g dm while they ranged between 0.01-0.47 mg/100 g dm for zinc. The highest iron bioaccessibility was observed for all cereals from Chiweshe and Chiredzi while the highest zinc bioaccessibility of 0.47 mg/100 g dm was observed for pearl millet from Hwedza.

4.4.5 Estimation of fermented cereals contribution to iron and zinc absolute requirements

The contribution that the porridges can make towards the absolute requirements and RDA of iron ranged between 25-411% (median of 93% for all fermented slurries and 62% excluding the cases where extrinsic soil iron was present) and 33-514% (median of 110% for all fermented slurries and 58% excluding the cases where extrinsic soil iron was present), respectively, while it ranged from 0.5-23% (median of 5%) and 11-35% (median of 16%), respectively for zinc (**Figure 4.2**). The highest contributions to absolute requirements for iron were obtained from Chiweshe and Chiredzi fermented slurries where cereal grains were contaminated by soil iron with lower contributions observed for maize and finger millet fermented slurries (27-62%). About 33% of the porridges could not meet at least 75% of the RDA of iron while none of the porridges could meet 75% of the RDA of zinc. The porridges that could not meet 75% of the iron RDA were the maize porridges from Mutoko and Hwedza, finger millet porridge from Mutoko, Hwedza and Victoria Falls and the pearl millet from Mutoko.

Table 4.6: Soluble non dialyzable and bioaccessible (dialyzable) iron (%) of fermented slurries from different locations in Zimbabwe

	Chiweshe	Chiredzi	Mutoko	Hwedza	Vic Falls	SEM	P value
				SND			
Maize	$31.0^{b,A,B}$	$14.5^{a,A}$	$39.5^{B,C}$	$65.0^{b,C}$	ns	4.98	0.001
Sorghum	$23.3^{a,b,A}$*	$27.2^{b,A}$	36.5^{A}	$35.8^{a,A}$*	$60.3^{b,B}$	4.44	0.001
P/millet	ns	$10.6^{a,A}$	$42.3^{B,C}$	$51.5^{a,b,C}$	$28.8^{b,A,B}$	4.39	0.001
F/millet	$21.7^{a,B,C}$	$15.0^{a,b,A}$	29.2^{C}	$47.6^{a,b,D\S}$	$56.1^{b,D}$	2.52	< 0.001
SEM	1.56	2.68	5.99	4.91	3.16		
P value	0.039	0.010	0.488	0.031	0.001		
				Bioaccessible (dialyzable)			
Maize	$16.0^{a,B}$	$3.29^{a,A}$	$5.36^{a,A}$	$14.10^{c,B}$	ns	0.79	< 0.001
	(2.69)	(1.28)	(0.32)	(0.45)			
Sorghum	$14.6^{a,B}$*	$12.0^{b,A,B}$	$18.0^{b,B}$	$8.12^{a,b,A}$*	$12.9^{b,A,B}$	1.28	0.004
	(3.78)	(3.98)	(0.85)	(0.66)	(0.72)		
P/millet	ns	$3.28^{a,A}$	$2.77^{a,A}$	$7.08^{a,A,B}$	$9.15^{a,b,C}$	1.27	0.027
		(1.63)	(0.25)	(0.96)	(1.44)		
F/millet	$26.1^{b,C}$	$8.86^{b,B}$	$7.07^{a,c,A,B}$	$12.6^{c,B\S}$	$4.99^{a,A}$	1.49	< 0.001
	(3.18)	(3.45)	(0.32)	(0.60)	(0.27)		
SEM	1.55	0.89	1.30	1.33	1.14		
P value	0.004	< 0.001	< 0.001	0.006	0.008		

*Sorghum type is red variety while the rest are white varieties. § Finger millet type is white variety while the rest are red varieties. p/millet: pearl millet, f/millet: finger millet, SND: soluble non dialyzable, SEM: standard error of mean. Values with different small superscript letters within columns are significantly different. Values with different capital superscript letters across rows are significantly different. Values in parenthesis are mean values of bioaccessible (dialyzable) contents of iron (mg/100 g dm), $p < 0.05$, n=3, ns – no sample.

Table 4.7: Soluble non dialyzable and bioaccessible (dialyzable) zinc (%) of fermented slurries from different locations in Zimbabwe

	Chiweshe	Chiredzi	Mutoko	Hwedza	Vic Falls	SEM	P value
				SND			
Maize	$21.4^{A,B}$	15.6^{A}	$12.8^{a,A}$	$29.7^{a,b,B}$	ns	2.47	0.006
Sorghum	21.4^{A}*	22.5^{A}	$22.3^{b,A}$	$30.9^{b,A}$*	$53.5^{b,B}$	3.01	< 0.001
P/millet	ns	11.8^{A}	$32.6^{c,B,C}$	$37.3^{b,C}$	$26.7^{a,B}$	1.67	< 0.001
F/millet	15.9	16.3	$16.7^{a,b}$	$22.7^{a,§}$	22.1^{a}	1.51	0.050
SEM	2.01	2.93	2.00	1.69	2.44		
P value	0.167	0.157	0.001	0.002	< 0.001		
				Bioaccessible (Dialyzable)			
Maize	$6.03^{b,B}$ (0.14)	$11.4^{a,b,B}$ (0.27)	$0.79^{a,A}$ (0.01)	$1.67^{a,A}$ (0.05)	ns	1.67	0.014
Sorghum	$1.86^{a,A}$* (0.01)	$11.9^{b,C}$ (0.27)	$8.92^{b,B}$ (0.17)	$0.74^{a,A}$* (0.01)	$1.21^{a,A}$ (0.02)	0.44	< 0.001
P/millet	ns	$4.11^{a,B}$ (0.16)	$2.07^{a,A}$ (0.09)	$10.8^{b,C}$ (0.47)	$1.95^{b,A,B}$ (0.09)	0.53	< 0.001
F/millet	$3.63^{a,b,A}$ (0.07)	$9.04^{a,b,C,D}$ (0.22)	$6.35^{b,B,C}$ (0.1)	$12.8^{b,D§}$ (0.16)	$0.45^{a,A}$ (0.01)	0.79	< 0.001
SEM	1.31	1.35	0.67	0.70	0.21		
P value	0.038	0.021	< 0.001	< 0.001	0.006		

*Sorghum type is red variety while the rest are white varieties. § Finger millet type is white variety while the rest are red varieties, f/millet: finger millet, p/millet: pearl millet, SND: soluble non dialyzable, SEM: standard error of means. Values with different small superscript letters within columns are significantly different. Values with different capital superscript letters across rows are significantly different. Values in parenthesis are mean values of bioaccessible (dialyzable) contents of zinc (mg/100 g dm), $p < 0.05$, n=3, ns – no sample.

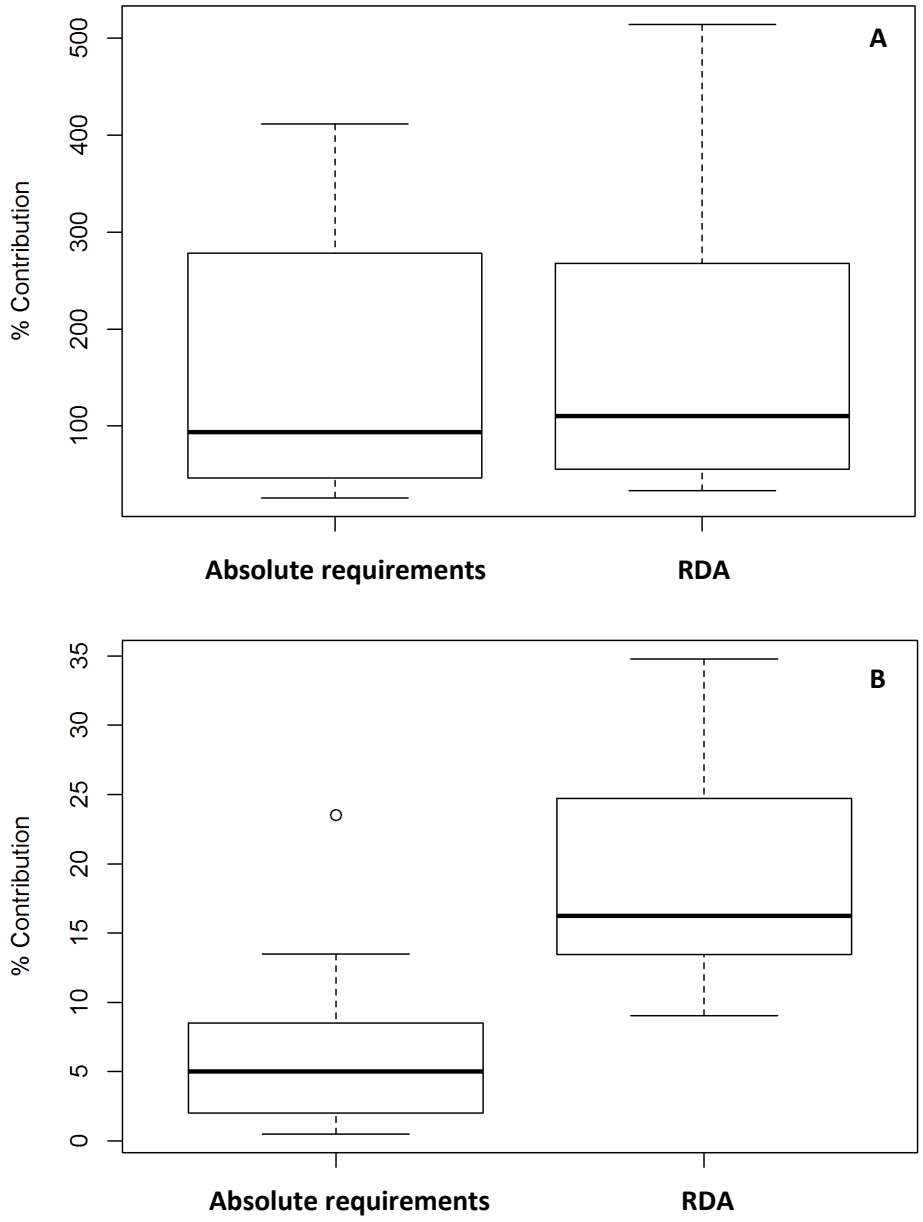

Figure 4.2: Contribution (%) of fermented porridges towards the iron (A) and zinc (B) absolute requirements and recommended dietary allowance

RDA: recommended dietary allowance. Absolute requirement is defined as the requirement for growth and basal losses. Maximum consumption of 100 g portion of porridge, 20% dry matter, 3 times a day was considered. Absolute requirement for iron is 0.58 mg/day for children between the ages 1-3 years while for zinc it is 1.2 mg/day for the same age group. Recommended dietary allowance for zinc is 8.3 mg/day (assuming 15% bioaccessibility) for iron it is 5.8 mg/day (assuming 10% bioaccessibility) (World Health Organization, 2004).

4.5 Discussion

The objective of the present study was to explore the bioaccessibility of iron and zinc of fermented slurries originating from different locations of Zimbabwe. The influence of fermentation on mineral binders (PA, PC and CT) and subsequent mineral bioaccessibility was estimated using *in vitro* dialyzability.

The total PC of plants is influenced by environmental conditions such as sun exposure, type of soil and amount of rainfall received (Kumari et al., 2017). A study by Kumari et al. (2017) showed large differences in the soluble and bound phenolic contents of millet grains and these differences were attributed to the differences in variety and location where the millets were grown. These observations are consistent with our findings as the observed levels of PC were influenced by location and cereal grain type (Table 4.2). Fermentation caused a general increase in the soluble PC and a decrease in the bound PC except for red sorghum where the opposite occurred. The changes occurring to PC after fermentation can be a result of the low pH which caused the abstraction of hydride ions with consequential reorganization of PC and also possible reduced extractability of PC due to self-polymerization and interaction with macromolecules (Towo et al., 2006). Furthermore, depolymerization of PC into low molecular weight PC can occur, thereby increasing the soluble fraction of the PC (Taylor and Duodu, 2015). Such observations have also been reported after fermentation of finger millet (Gabaza et al., 2016) and tef (Shumoy et al., 2017). The production of enzymes with ability to metabolize PC such as glucosidase, phenolic acid reductase and phenolic acid decarboxylase from LAB in particular *Lactobacillus plantarum*, *Lactobacillus case*i, *Lactobacillus fermentum* and *Lactobacillus reuteri* is responsible for changes in PC during fermentation (Svensson et al., 2010). Indeed, *L.casei*, *L. fermentum*, *L. reuteri* and *L. plantarum* have been identified in several sorghum and millet fermented foods from Africa (Gabaza et al., 2017a).

The high contribution of soluble PC to total PC for sorghum (36%) likely emanated from the CT which were multiple times higher than in finger millet (Table 4.3). The behavior of the soluble PC fraction of red sorghum after fermentation was markedly different from that of finger millet which suggests differences in the structure of their CT. Sorghum CT bind with macromolecules such as proteins after fermentation such that they become less extractable and assayable (Dlamini et al., 2007). This phenomenon was observed from the reduction of soluble PC after fermentation in sorghum but not in finger millet. In contrast, an increase in CT was observed with the vanillin assay for both red sorghum and red finger millet. As catechin reacts positively in the vanillin assay, it can be inferred that part of what is measured as CT is catechin and its oligomers. In fact, we suspect that most of what is measured

as CT in finger millet is catechin and its oligomers, as up to 84% of the soluble PC in finger millet has been found to be mainly catechin (Chandrasekara and Shahidi, 2011; Gabaza et al., 2016).

A reduction of PA after fermentation of maize, sorghum and millets has been widely reported. During fermentation, there is production of microbial phytases and also activation of endogenous phytases due to the low pH environment causing the degradation of PA (Towo et al., 2006). However, the activation of endogenous phytases can be considered non-significant in the non-Triticeae type of cereals used in our study as they are known to possess very little endogenous phytase activity (Brinch-Pedersen et al., 2014). Hence the phytases are assumed to be largely derived from the microorganisms active during fermentation. Since PA was reduced after fermentation in all cereals as shown in Table 4.4, further optimization of the fermentation process may be needed to ensure almost complete degradation of PA. In addition, as degradation of PA in some cereals was much higher than in others, microorganisms involved in the fermentations may differ.

As expected, there were large variations in the iron and zinc contents of the cereals attributed to the differences in the locations, type of cereal and variety of cereal (Table 4.5). The high iron contents observed in Chiweshe and Chiredzi could be a result of contamination from extrinsic sources. A positive correlation of aluminum and iron in cereal grains and flours suggests soil contamination (Gibson et al., 2015; Siyame et al., 2013), which was also observed in the present study (R^2 = 0.87; individual data not shown) alluding to some soil contamination for most of the cereal grains. Threshing of cereal grains is associated with soil contamination and all cereals used in the present study, except maize, went through a traditional threshing process. The level of soil contamination in cereal flours from other locations may be negligible as cultural threshing practices may highly influence iron contamination. In some areas, threshing is done on a bare rock or in sacks where contact with soil is limited, while in others it is done on a surface exposed to soil such that there is a potential for extensive contamination. High iron content in all cereals from Chiredzi was matched with high aluminum contents including maize, which is not associated with threshing, implying that these samples were definitely contaminated by soil. The fact that maize was also contaminated shows that threshing is not the only process introducing extrinsic iron from soil. Soil contamination could have also occurred during storage as it was observed in Chiredzi that cereal panicles were stored in mud houses where they were in contact with soil soon after harvest. In some cases, cereal panicles are also dried on the ground thereby exposing the cereal grains to contamination. The extrinsic iron of cereals from Chiweshe is not certain if it originated from soil as the iron content in maize was not correlated with the aluminum content. The source of contamination of these samples is thus unclear but is unlikely to be from soil.

The zinc contents of the cereals were lower than the recommended level of 4-6 mg/100 g dm as shown in Table 4.5 (Pfeiffer and McClafferty, 2007) and this is because Zimbabwean soils are known to be zinc deficient (Manzeke et al., 2012; Tagwira, 1991). Analysis of the soils from the locations in the present study revealed low available zinc contents of < 1.5 mg/kg (data not shown) which is the minimum requirement for soils to have sufficient zinc content in grains (Dobermann and Fairhurst, 2000). On the other hand, available iron content of the soils was beyond the recommendation of 5 mg/kg (Dobermann and Fairhurst, 2000; Nikolic et al., 2016) implying that soil iron content was adequate but other factors such as absorption of iron from soil to crop could limit the iron content in cereals such as finger millet which consistently had lower iron content.

According to the observed results from this study, the iron and zinc bioaccessibility of fermented cereals seems to be dependent on mainly presence of extrinsic iron and type of cereal flour and these factors will be discussed in detail. Clear differences were observed between the iron bioaccessibility of the two locations, Chiweshe and Chiredzi, where the cereals were contaminated with extrinsic iron. Cereals from Chiweshe had iron bioaccessibility of 14.6-26% in contrast to Chiredzi cereals with bioaccessibility of 3.28-12.0% (Table 4.6). Higher iron bioaccessibility from Chiredzi was anticipated because of the lower PA: Fe ratios which were in the vicinity of 1 and even lower than 0.4 for pearl millet. According to Gibson et al. (2015), soil pH plays a crucial role in determining the bioaccessibility of iron in soil contaminated cereals with acidic soils (median pH 5.2) being of higher iron bioavailability compared to calcareous soils (median pH 7.8) possibly because of their buffering capacity which can help to maintain an acidic environment during gastric digestion thereby enhancing the reduction of Fe^{3+} to Fe^{2+}. However, all the soils where the grains were collected from had an acidic pH ranging from 4.67-5.70 (data not shown) hence soil pH may not be the only determinant of iron bioaccessibility in samples contaminated with soil. Low iron bioaccessibility of less than 5% observed for sorghum and pearl millet from Chiredzi was also reported for iron contaminated sorghum and millet samples, although this contamination was associated with milling equipment (Icard-Vernière et al., 2013). Ferric oxides and ferric hydroxides are generally considered the most common forms of extrinsic iron in foods and are of low bioavailability (not more than 1.5%) (Harvey et al., 2000). Although the exact origins of the contaminant iron from Chiweshe could not be ascertained, our study indicates that certain types of contaminant iron are potentially bioaccessible and might have a potential of improving the iron status of communities involved.

The type of cereal clearly plays an important role in determining the bioaccessibility of iron and zinc. A distinct difference in both iron and zinc bioaccessibility existed between high PC or CT cereals with low PC cereals. Red sorghum from Chiweshe had a bioaccessibility of 14%, which, as described before, was most probably due to the highly bioaccessible contaminant iron. Analogous to these results is the

higher iron bioaccessibility of white finger millet compared to its red counterparts (Table 4.6). The effect of PC was also observed in relation to zinc bioaccessibility with zinc bioaccessibility of red sorghum not exceeding 2% compared to up to 11.9% for white sorghum. The same effect was also observed between white and red finger millet. White sorghum from Victoria Falls had lower zinc bioaccessibility of 1.21% which was comparable to that of the red sorghum. This could be due to the high PC found in this cereal which was almost double that of its white counterparts. Several studies have shown the low bioaccessibility of iron and zinc in high PC and CT cereals despite even complete dephytinization (Baye et al., 2015; Cercamondi et al., 2014b; Towo et al., 2006). Our results therefore, further strengthen the assertion that PC in cereal grains have high mineral binding power such that levels of PA alone may not be the only predictor of the bioaccessibility of cereal and cereal products.

The present study also revealed that the structure of PC in some cereals may be more important than the total PC. The iron and zinc bioaccessibility of red finger millet was comparable to that of the low PC cereals such as maize and white sorghum, and even higher than that of pearl millet. Although pearl millet had a lower PC than either red sorghum and red finger millet, its iron and zinc bioaccessibility was consistently low suggesting that pearl millet may contain certain PC which strongly chelate iron and zinc (Table 4.6 and 4.7). Furthermore, pearl millet had a higher fraction of soluble PC than other cereals and these soluble PC could be the key to its low iron and zinc bioaccessibility. It is a well-established fact that PC with catechol and galloyl groups bind minerals with negative implications on their bioaccessibility (Brune et al., 1991). However, Hart et al. (2017) revealed the ability of some PC such as kaempferol, without the aforementioned groups to bind iron, inducing in some cases a positive effect on bioavailability. The authors revealed that some PC promote iron uptake by Caco-2 Cells possibly due to their ability to reduce Fe^{3+} to Fe^{2+}, although this ability is much lower than that exhibited by ascorbic acid. The study by Hart et al., (2017) was performed using phenolic compounds extracted from black bean. Likewise, studying the influence of phenolic extracts from maize, sorghum and millets on iron and zinc bioaccessibility may provide some valuable information pertaining to their potency as mineral binders. The major PC present in the SND+D fraction may distinguish between enhancing and inhibitory PC. Pearl millet from Hwedza had higher iron and zinc bioaccessibility than from other locations. While the pH of other fermented pearl millet was between 4.39-4.50, this pearl millet had a pH of 5.33 after 26 h of fermentation. It is thus conceivable that pH buffering capacity may also play a crucial role in determining the bioaccessibility of fermented cereals. The buffering capacity of the fermented food is particularly important for iron as it can influence the iron redox reactions which are critical for iron absorption.

No clear trend was detected in terms of the effect of PA on iron bioaccessibility as other parameters such as presence of extrinsic iron and PC seemed also involved in determining iron bioaccessibility. Likewise, only the samples that were contaminated with extrinsic iron had lower PA: Fe ratios such that the effect of PA on iron bioaccessibility was not apparent. Low PC cereals from Chiredzi which had the lowest PA levels had higher zinc bioaccessibility (up to 11%) than others with bioaccessibility mostly in the vicinity of 5% (Table 4.7). Complete dephytinization of PA in these types of cereals may potentially improve the bioaccessibility of zinc. It is evident that the determinants of iron and zinc bioaccessibility are multifaceted and different and may include the microbiota of the fermented cereals as well. Other factors that could also influence the iron and zinc bioaccessibility of the fermented cereals are related to the interaction between the soluble and insoluble food components that could lead to competition for complexation of minerals between soluble and insoluble mineral binders and the kinetics of release of both the minerals and the mineral binders from the fermented slurries.

An exploration of the bioaccessibility (%) of iron and zinc is valuable to gain information on the total sum effects of mineral binders in a given cereal matrix. However, the levels of the bioaccessible iron and zinc are more important in determining their contribution to iron and zinc nutrition in vulnerable populations. Although the bioaccessibility of extrinsic iron from Chiredzi was low, it provided way more bioaccessible iron than other cereals from other locations. In fact, the contribution of iron towards the absolute requirement was beyond 100% in all cases where extrinsic iron was involved (Figure 4.2). Higher contribution of iron towards the absolute requirement (25-148%) was observed for pearl millet than for other cereals. It was proposed that when estimating the contribution that the bioaccessible minerals can make towards the absolute requirements, it is more reliable to consider the existence of a positive effect rather than the magnitude of that effect (Fairweather-Tait et al., 2005). In that respect, fermented cereals where extrinsic iron was involved could contribute five times more iron to the absolute requirements than other cereals, while pearl millet fermented slurries could contribute almost twice of the absolute requirements compared to that of other cereals. This purports that contaminant iron may make a meaningful contribution to iron nutrition and also demonstrates that strategies to improve iron nutrition could target pearl millet as it holds much potential for improving iron nutrition. Maize is the main cereal consumed in more than 50% of households in Zimbabwe. More incorporation of small seeded grains such as pearl millet into the diet of people may help to achieve the objective of reducing iron deficiency. Dissimilar results were observed for zinc as none of the fermented cereals could provide close to 50% RDA (Figure 4.2). Pertaining to the absolute requirements of zinc, only pearl millet from Hwedza could contribute more zinc to the absolute requirement than other cereals. Observations of a higher zinc inadequacy compared to iron have been

reported (Kruger et al., 2015). In a cross-sectional study among women of child bearing age in Malawi, a risk of zinc deficiency of > 90% compared to < 15% for iron was reported (Siyame et al., 2013).

Estimations of the contributions fermented cereals can make towards the absolute requirements and RDA are based on the maximum possible levels of intake; much lower contributions are probably being experienced in real life situations. We used an *in vitro* approach to estimate bioavailability hence caution should be exercised when interpreting these results. In addition, porridges have to undergo a final cooking process to make thin and thick porridges that are widely consumed by the communities in Zimbabwe and other parts of Southern Africa.

4.6 Conclusions

Iron and zinc bioaccessibility was influenced by the total interactions of mineral binders within their matrix. There was a large variation in iron and zinc bioaccessibility from different locations and also from different cereal grain types. Contaminant iron may positively contribute to iron intake in communities where it is high. Zinc bioaccessibility was low across all locations so concerted efforts are needed to improve both the bioaccessibility and total zinc contents in cereals. Fermentation showed some positive effect towards iron and zinc bioaccessibility and its common use in the preparation of complementary porridges in developing countries needs to be encouraged and further studied to allow optimization.

Chapter 5

High-throughput partial 16S rRNA gene sequencing of Zimbabwean maize, sorghum and millet fermented slurries

Chapter 5: High-throughput partial 16S rRNA gene sequencing of Zimbabwean maize, sorghum and millet fermented slurries

5.1 Abstract

Maize, sorghum and millet fermented porridges are important as weaning and complementary foods in Africa. The objective of the present study was to gain some insights into the bacterial communities of fermented slurries prepared from maize, sorghum and millets originating from different locations in Zimbabwe, and prepared either at household or laboratory level. A deep sequencing approach targeting the hypervariable V4 region of the 16S rRNA gene was used and yielded about 100,000 sequences per sample. *Lactococcus* dominated all the fermented slurries, flanked by other lactic acid bacteria such as *Weissella*, *Leuconostoc* and *Enterococcus*. *Enterobacteriaceae* detected in the water samples persisted throughout all the fermented cereals. Other sub-dominant bacteria identified in the fermented slurries included *Aeromonas*, *Pseudomonas* and *Acinetobacter*. In addition, some Proteobacteria, Actinobacteria and Bacteroidetes associated with the raw materials and environment were also detected. Fermented slurries could not be differentiated based on their origin nor on the type of fermentation, but clear differences were observed between red sorghum fermented slurries and slurries prepared from other cereal flours. A thorough understanding of the functional capacities of the microbiota in African fermented slurries is highly needed in order to steer the fermentation for the production of standard, safe and nutritious fermented products.

Keywords: maize, sorghum, millet, bacterial communities, sequencing

Molly Gabaza, Marie Joossens, Margo Cnockaert, Maud Muchuweti, Katleen Raes, Peter Vandamme (2017). Lactococci dominate the bacterial communities of fermented maize, sorghum and millet slurries in Zimbabwe. In preparation for submission.

5.2 Introduction

Fermented cereal products constitute a vital part of the African diet providing a low cost, energy efficient method of food processing and preservation. A wide range of fermented cereal products are consumed in Africa and include both thin and thick porridges, alcoholic and non-alcoholic beverages, and bread like products (Blandino et al., 2003; Gabaza et al., 2017). These products are important as dietary staples, complementary and weaning foods, refreshments, and condiments, and are also essential for cultural ceremonies. The production of fermented foods is typically done at household level and in small production units under rudimentary conditions, which pose hygienic and toxicological risks (Achi and Ukwuru, 2015; Holzapfel, 2002). Additionally, the quality of the products is uncontrollable and unpredictable such that the final quality and safety is highly variable.

Some of the African fermented cereal products, particularly the porridges and gruels used as weaning and complementary foods, need improvements in terms of their safety and nutritional content. The use of functional starter cultures offers a great potential and has been successful in the production of a high energy pearl millet gruel after using a starter culture with high amylolytic activity (Songré-Ouattara et al., 2009). Starter cultures with the ability to degrade mineral binders for improved mineral bioavailability among others are also urgently needed. A prerequisite to the production of starter cultures is a thorough characterization of the microbial diversity of the fermented foods concerned. The functional capacity of different microbial consortia found in each product (Oguntoyinbo et al., 2011) and the impact of process conditions and cereal substrates on the microbial diversity needs to be carefully studied.

The emergence of next generation sequencing methods brought new opportunities to the study of food fermentations as they are fast, cost effective and give a deeper understanding of the microbial ecology of fermented foods (Bokulich and Mills, 2012; Van Hijum et al., 2013). While the bacterial communities of African fermented foods has mainly been studied using traditional molecular techniques (Assohoun-Djeni et al., 2016; Madoroba et al., 2011; Mukisa et al., 2012; Oguntoyinbo et al., 2011), to date, only the microbial community of African pearl millet slurries has been described comprehensively through the application of modern day sequencing techniques (Humblot and Guyot, 2009). Sour porridge is an important type of fermented porridge in Zimbabwe that is mainly consumed by children as a weaning and complementary food, and is made from maize, sorghum and millets. The bacterial diversity of sour porridge is not known. We therefore applied 16S rRNA amplicon sequencing to decipher the bacterial communities of different types of fermented cereals typically used in the preparation of porridge. The objective of the present study was to describe the bacterial communities of fermented slurries from maize, sorghum and millets and to carry out a comprehensive comparative

analysis of the bacterial diversity based on type of fermentation, type of cereal substrate and origin of fermented cereal.

5.3 Materials and methods

5.3.1 Materials

The finger millet grains used in the preparation of household fermented slurries were provided by farmers from Ushe communal area, in Hwedza, Zimbabwe and were harvested during the 2013/2014 season. Cereals (maize, sorghum, pearl millet and finger millet) were provided by farmers from five locations from Zimbabwe and were harvested during the period 2015/2016. Power Microbiome™ Isolation kit and MagAttract suspension G was procured from Qiagen, Belgium. The Qubit™ dsDNA assay kit was obtained from Life Technologies.

5.3.2 Methods

Two studies were performed: one based on household fermented finger millet slurries sampled from a typical rural setting of Zimbabwe described in chapter 3 and a second study based on laboratory fermented cereals originating from different rural parts of Zimbabwe described in chapter 4.

5.3.2.1 Household fermented slurries

The sampling, preparation and collection of household fermented slurries is described in Chapter 3. Four varieties of finger millet were used in the study i.e. RV1-red variety 1, RV2-red variety2, WV1-white variety 1, WV2-white variety 2. Two types of fermented slurries were prepared for each variety i.e. SFS-spontaneously fermented slurry and BFS-backslopped fermented slurry. **Figure 5.1** shows an overview of the preparation of the fermented slurries.

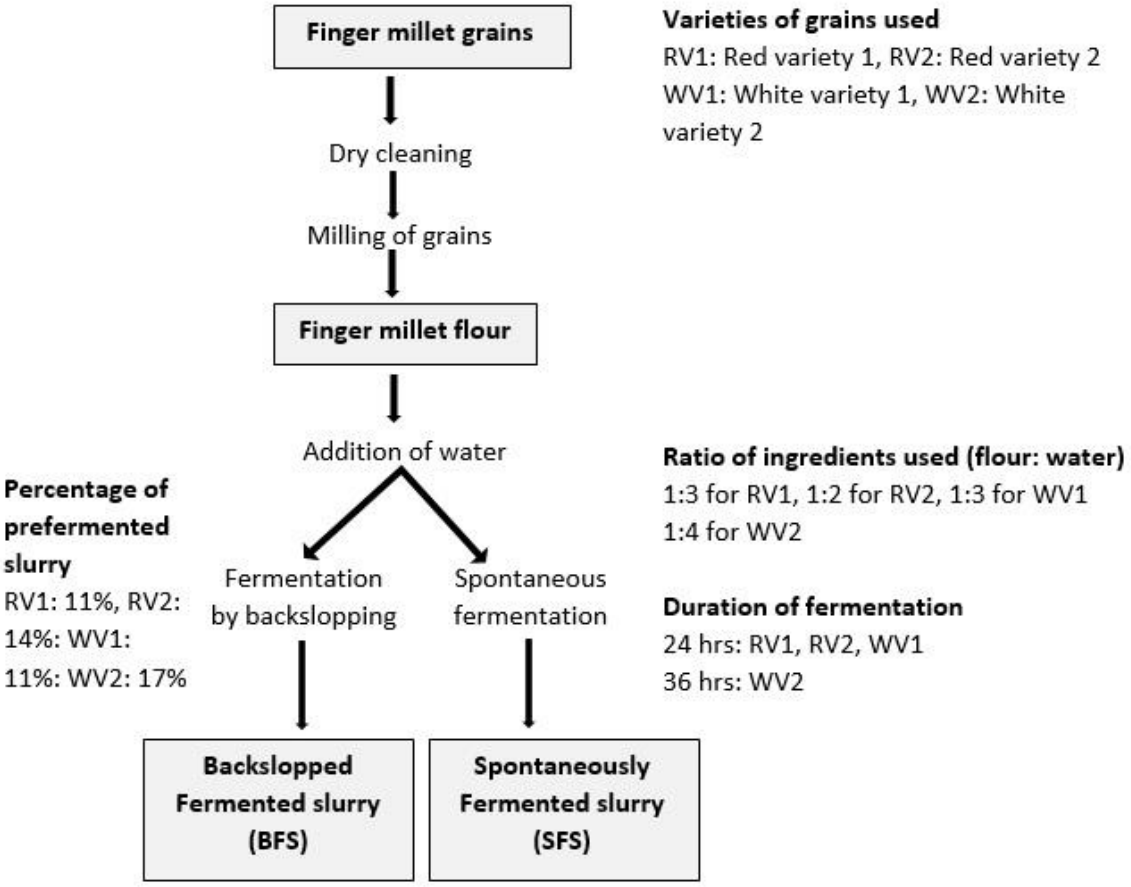

Figure 5.1: Schematic showing the flow chart for the preparation of fermented slurries

RV1: red variety 1, RV2: red variety 2, WV1: white variety 1, WV2: white variety 2, SFS: spontaneously fermented slurry, BFS: backslopped fermented slurry.

5.3.2.2 Laboratory fermented slurries

Collection of cereal grains and water used in the preparation of laboratory fermented slurries originating from different locations is described in chapter 4 together with the description of the preparation process of the fermented slurries. Cereal grains (maize, sorghum, pearl millet and finger millet) were collected from five locations, namely Chiweshe, Chiredzi, Mutoko, Hwedza and Victoria falls.

5.3.2.3 Extraction of DNA

DNA was extracted from fermented cereals and water. An aliquot of thawed fermented slurry (50 mL) was first centrifuged at 1000 x g for 10 min. Then the supernatant was collected and subjected to another centrifugation at 1000 x g for 10 min to remove the starchy pellet and then at 10000 x g for

10 min to pellet the bacterial cells. The pellet from the first centrifugation was washed with physiological water followed by the centrifugation steps as described before in order to collect as much bacterial cells as possible. This process was repeated three times and the bacterial cells were pooled. Water samples were centrifuged at 10000 x g for 20 min to pellet the bacterial cells. DNA from the obtained bacterial cells was extracted using the Power Microbiome™ Isolation kit according to the manufacturers protocol with an additional lysis step at 90°C for 10 min after the fourth step. Purification of the DNA was done by suspending 5 µL of 3 M sodium acetate and 125 µL absolute ethanol to 50 µL of extracted DNA and precipitating the DNA overnight at -20 °C. Centrifugation was performed at 13000 x g for 30 min after which the DNA was washed twice with 500 mL of ice-cold ethanol (75%). The DNA was air dried and suspended in DNase free water. To ensure the DNA conformed to the required specification for sequencing, the quality of the DNA was assessed with Nanodrop (NanoDrop ND-1000, Thermo Fischer) (OD260/280: 1.8-2.0), agarose gel electrophoresis (no DNA degradation and RNA contamination) and the concentration was measured using Qubit 3.0 Fluorimeter (Life Technologies, Carlsbad, CA, USA) (> 5 ng/µL and amounting to > 100 ng). Out of 81 DNA samples, 15 samples did not meet the quality criteria and were further purified by suspending the DNA in 100 µL high salt Tris-EDTA buffer and incubating for 30 min at 60 °C, followed by adding 5 µL of MagAttract suspension G and 120 µL of absolute ethanol. This mixture was vortexed gently and was incubated for 5 min at room temperature. The tubes were placed on a magnetic rack in order to separate the beads binding the DNA from the suspension. The beads were washed three times with wash buffer (30% TE and 70% ethanol) and air dried at room temperature for 10 min. DNase free water was then added to the beads to suspend the DNA and this was incubated for 5 min at 60 °C followed by separation of the beads from the DNA on the magnetic rack.

5.3.2.4 16S rRNA amplicon library preparation and amplicon sequencing

DNA samples were shipped to Novogene Bioinformatics Technology Co., Ltd, Hong Kong (https://en.novogene.com/next-generation-sequencing-services/microbial-genome/amplicon-sequencing/) where library preparation and amplicon sequencing was performed. Amplification of the 16S rRNA gene was performed by targeting the hypervariable V4 region using the primers 515F and 806R tailed with illumina adapters with indexing barcodes, to generate fragment sizes of approximately 292 bp. Paired-end sequencing was carried out on HiSeq PE250 to generate sequences of high quality (Q30 > 80%) and sequencing depth of at least 30000 raw tags per sample.

5.3.2.5 Sequence analysis

Demultiplexing, denoising and removal of chimeric sequences was performed by means of the LotuS pipeline (Hildebrand et al., 2014) after which the reads were binned into operational taxonomic units (OTU's) based on 97% OTU similarity. The taxonomic origin of each OTU was determined by blasting the sequences on different databases i.e. Ribosomal Database Project, PR2, Green genes and Silva. There were minor differences in OTU assignment among the different databases. The assignment derived from the Ribosomal Database Project was considered. Alpha diversity (Chao1, Shannon and Simpson diversity) indices were calculated to estimate the bacterial biodiversity in each sample. The percentage of bacterial OTU's in each sample were calculated as a proxy to their relative abundances. Based on the relative abundances, bacterial genera were classified into three different population groups where < 1% were considered as rare genera, 1 to < 10% as sub-dominant genera and finally the genera with relative abundance of > 10% as dominant (Nam et al., 2012).

5.3.2.6 Statistical analysis

The relative abundances of taxa present in at least 25% of the samples and the diversity measures were compared using the Kruskal Wallis test and q-values < 0.05 after Bonferroni correction for multiple testing were considered significant. In household fermented slurries, bacterial populations in spontaneous and backslopped fermentation of each finger millet variety were compared. In laboratory fermented slurries, comparisons of bacterial populations were made based either on cereal type or location. Also bacterial populations of spontaneously fermented finger millet household slurries (white varieties) and laboratory fermented finger millet slurries from Hwedza were compared. Principal coordinate analysis (PCoA) using weighted Unifrac distance matrix was carried out to visualize similarities and dissimilarities among all the samples sequenced i.e. water, household and laboratory fermented slurries. Statistical analysis were based on two independent samples for the household fermented slurries and three independent samples for the laboratory fermented slurries. Data in tables is shown as mean ± standard deviation. All statistical analysis were carried out using R version 3.3.2.

5.4 Results

5.4.1 Characteristics of household and laboratory fermented slurries

The pH of the household fermented slurries ranged between 3.89-4.62 whereas that of the laboratory fermented slurries ranged between 4.12-5.75 (**Table 5.1 and 5.2)**.

Table 5.1: pH of household finger millet fermented slurries after 24-36 h of fermentation

Sample	pH
RV1_SFS	3.96±0.09
RV1_BFS	3.89±0.08
RV2_SFS	4.62±0.01
RV2_BFS	4.28±0.10
WV1_SFS	4.19±0.01
WV1_BFS	4.28±0.10
WV2_SFS	4.42±0.04
WV2_BFS	4.46±0.28

RV1: red variety 1, RV2: red variety 2, WV1: white variety 1, WV2: white variety 2, SFS: spontaneously fermented slurry, BFS: backslopped fermented slurry, n=2.

Table 5.2: pH of laboratory fermented slurries after 26 h of fermentation

	Chiweshe	Chiredzi	Mutoko	Hwedza	Vic Falls
Maize	4.42±0.07	4.39±0.01	4.27±0.02	4.38±0.03	ns
Sorghum	5.66±0.03*	4.32±0.01	4.29±0.02	5.75±0.03*	4.12±0.10
Finger millet	4.45±0.04	4.64±0.02	4.48±0.06	4.73±0.08§	4.55±0.02
Pearl millet	ns	4.50±0.04	4.39±0.02	5.33±0.09	4.40±0.06

*sorghum type is red variety whilst the rest are white varieties, §finger millet type is white variety whilst the rest are red varieties, ns; no sample, n=3.

5.4.2 Diversity estimates of bacterial communities

After the quality control process which included the removal of sequences that could not be attributed to phyla within the domains of bacteria and archaea, and of sequences attributed to chloroplasts, a total of 6,861,665 high quality sequences were retained for the laboratory fermented slurries (an average of 114,361), 1,852,973 sequences for household fermented slurries (an average of 115,810) and 386,281 sequences for the water samples (an average of 96,570). Data of water samples from only 4 locations was available as the water sample from Chiweshe did not yield enough DNA material to meet sequencing requirements. Richness estimator (Chao1) and diversity indices (Shannon index and Simpson index) are presented in **Figure 5.2**. The observed number of OTUs ranged between 86 and 594 for the household fermented slurries, 57 and 610 for the laboratory fermented slurries and 464 and 1010 for the water samples. There were significant differences among the observed number of

OTUs (q < 0.05) with major differences between water samples and fermented cereals, and between red sorghum slurries and other types of fermented cereal slurries. The same trend was also observed when using the richness estimator Chao1, and in all samples the total number of OTUs estimated by Chao1 was slightly higher than the observed number of OTUs which covered an average of 70 to 80% of the estimated richness suggesting that there could be a few unseen OTUs. The highest diversity calculated using the Shannon index (q < 0.05) was found in the water samples, followed by the household fermented slurries and lastly the laboratory fermented slurries. In contrast, the Simpson-index (q < 0.05), which is a measure of both diversity and evenness, was highest in the water samples and household fermented slurries and lowest in the laboratory fermented slurries.

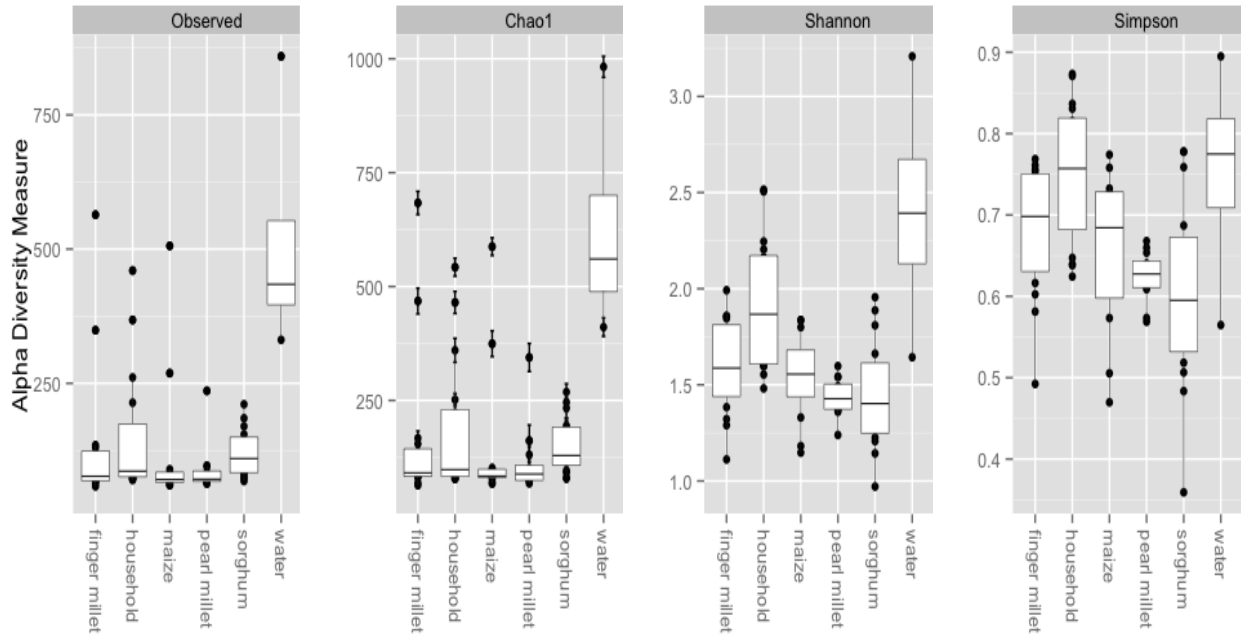

Figure 5.2: Diversity measures of bacterial 16S rRNA sequences in water, household fermented slurries and cereals (maize, sorghum, finger millet and pearl millet) from five locations of Zimbabwe

5.4.3 Bacterial communities of household fermented slurries

In general, the most abundant bacterial phylum in our study of household fermented slurries was the Firmicutes (72%) followed by Proteobacteria (27%). Other phyla represented very minor overall fractions. Of these, Actinobacteria represented the highest abundance (0.7%). The relative abundance at the phylum and genus level for the different finger millet household fermented slurries is shown in **Figure 5.3**. Finger millet household fermented slurries were dominated by *Lactococcus* (39%), unclassified *Enterobacteriaceae* (14%), *Weissella* (14%) and *Leuconostoc* (14%), while the subdominant genera included *Aeromonas* (6%), *Enterococcus* (3%), *Pseudomonas* (3%), *Lactobacillus* (2%) and

Acinetobacter (1%). An average of 5% of the total abundance was attributed to rare genera some of which were present in the water and belonged to Proteobacteria such as *Sphingomonas*, *Stenotrophomonas*, unclassified *Comamonadaceae*, *Acetobacter* and *Tolumonas*. Some Firmicutes commonly found in fermented cereals were also found among the rare genera and included *Pediococcus* and *Streptococcus*. Among more than 50 rare genera, *Curtobacterium* (Actinobacteria phylum) and *Sphingobacterium* (Bacteroidetes phylum) were the most dominantly detected.

5.4.4 Bacterial communities of laboratory fermented slurries

The laboratory fermented slurries were prepared using the flour and water from the different locations as these were expected to harbor the inocula for the fermentation and to be the main drivers of the fermentation. The relative abundance of the bacterial communities of the water and of the laboratory fermented slurries at the phylum and genus level is shown in **Figure 5.4 and 5.5**. Unlike in the fermented slurries, Proteobacteria constituted the major phyla in the water accounting for approximately 70% of the phyla, followed by about 23% Firmicutes, 4% Bacteroidetes and about 2% Actinobacteria, while about 1% of the phyla were considered as rare. Water from Mutoko had the highest abundance of Proteobacteria and the lowest abundance of other rare phyla while water from Victoria Falls had almost equal abundances of Firmicutes and Proteobacteria. Hwedza water contained the highest level of Actinobacteria. Four genera were classified as dominant and included unclassified *Comamonadaceae* (19%), *Acinetobacter* (18%), *Lactococcus* (15%) and unclassified *Enterobacteriaceae* (10%) while 9 genera were classified as subdominant i.e. *Aeromonas* (5%), *Flavobacterium* (3%), *Aquabacterium* (2%), *Enterococcus* (2%), *Leuconostoc* (2%), *Pseudomonas* (2%), an unclassified clade of Actinobacteria referred to as the Hgcl clade (Glöckner et al., 2000)(1%), *Hydrogenophaga* (1%) and *Dechloromonas* (1%). Most of the Firmicutes identified in water samples belonged to the order Lactobacillales and also included members of *Weissella*, *Lactobacillus* and *Streptococcus* among the rare genera. Water from different locations showed markedly different profiles in their bacterial communities at the genus level (**Figure 5.4**). Water from Chiredzi and Hwedza had more unclassified *Comamonadaceae* than the other two locations while water from Mutoko had more *Acinetobacter,* and water from Victoria Falls had more *Lactococcus* and unclassified *Enterobacteriaceae*. *Flavobacterium* was particularly high in Chiredzi water.

In general, laboratory fermented slurries, like the household fermented slurries showed dominance of Firmicutes (72%) and Proteobacteria (27%) (**Figure 5.5**). At the genus level, only *Lactococcus* (44%) and unclassified *Enterobacteriaceae* (29%) were considered dominant while the subdominant group comprised of *Leuconostoc* (8%), *Aeromonas* (7%), *Weissella* (3%), *Enterococcus* (3%) and *Pseudomonas*

(2%). Among more than 50 rare genera, the most dominantly detected included *Lactobacillus* (Firmicutes phylum), *Acinetobacter* (Proteobacteria phylum), *Tolumonas* (Proteobacteria phylum) and *Paenibacillus* (Firmicutes phylum). In the laboratory fermented slurries, the *Leuconostocaceae* family comprised of 73% *Leuconostoc* and 27% *Weissella* in contrast to 50% *Leuconostoc* and 50% *Weissella* in the laboratory fermented slurries.

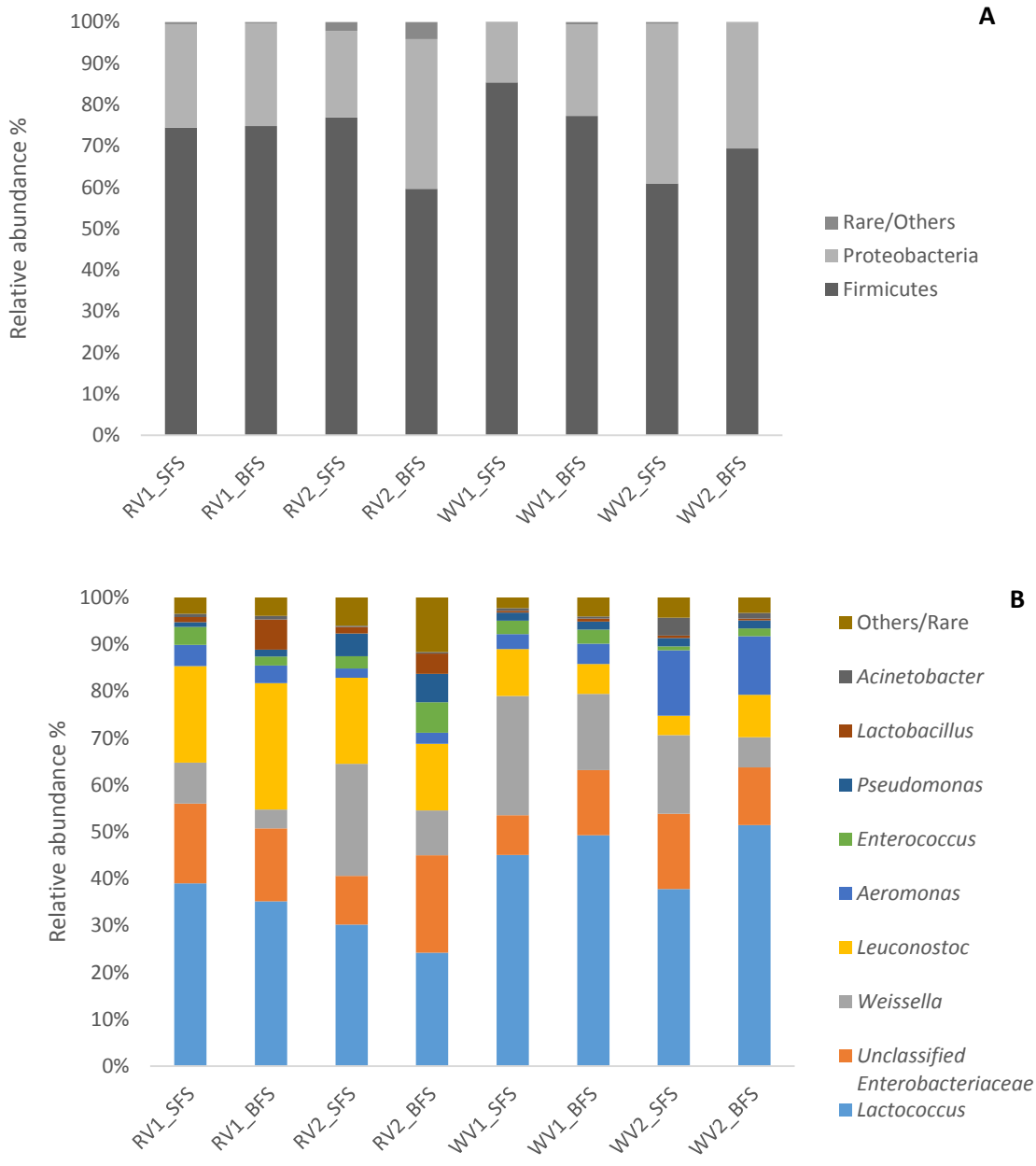

Figure 5.3: Relative abundance of bacterial communities of household fermented slurries at phylum (A) and genus level (B)

RV1: red variety 1, RV2: red variety 2, WV1: white variety 1, WV2: white variety 2, SFS: spontaneously fermented slurry, BFS: backslopped fermented slurry. Others/rare represent all phyla or genera with relative abundance of < 1%.

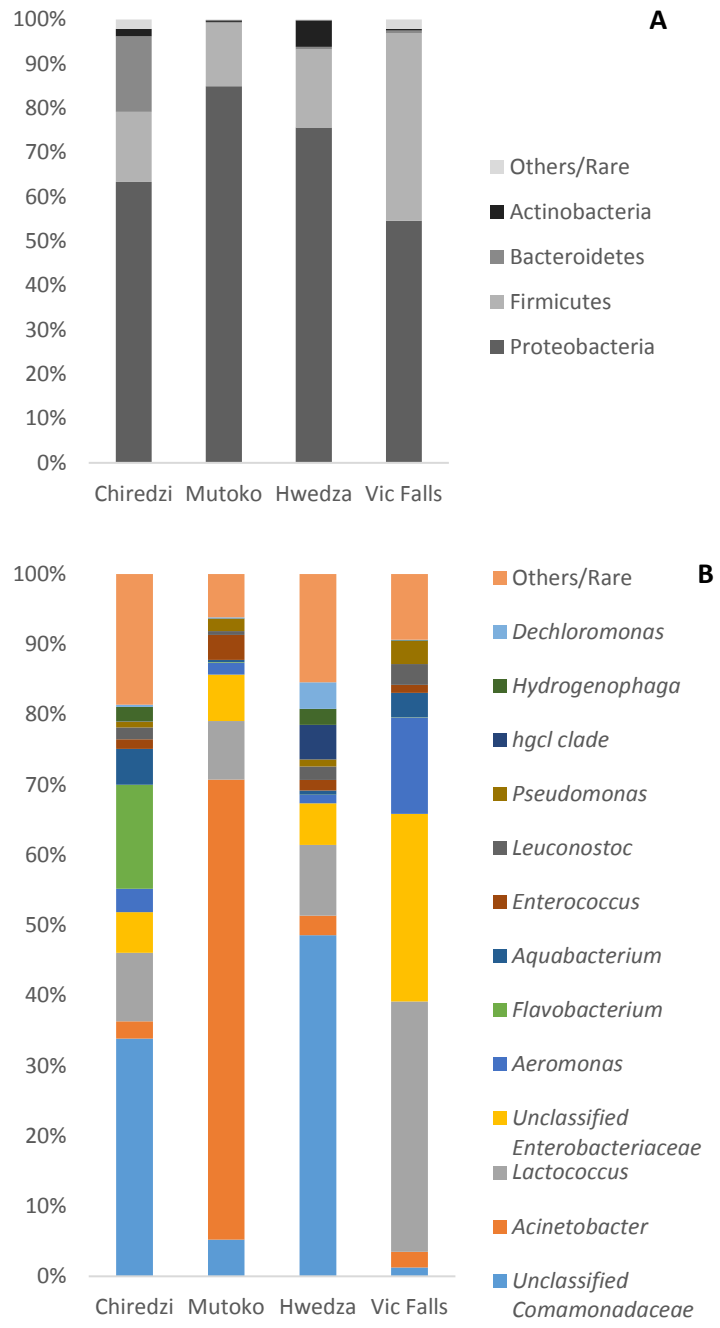

Figure 5.4: Relative abundance of bacterial communities of water at phylum (A) and genus level (B)

Others/rare represent all phyla or genera with relative abundance of < 1%.

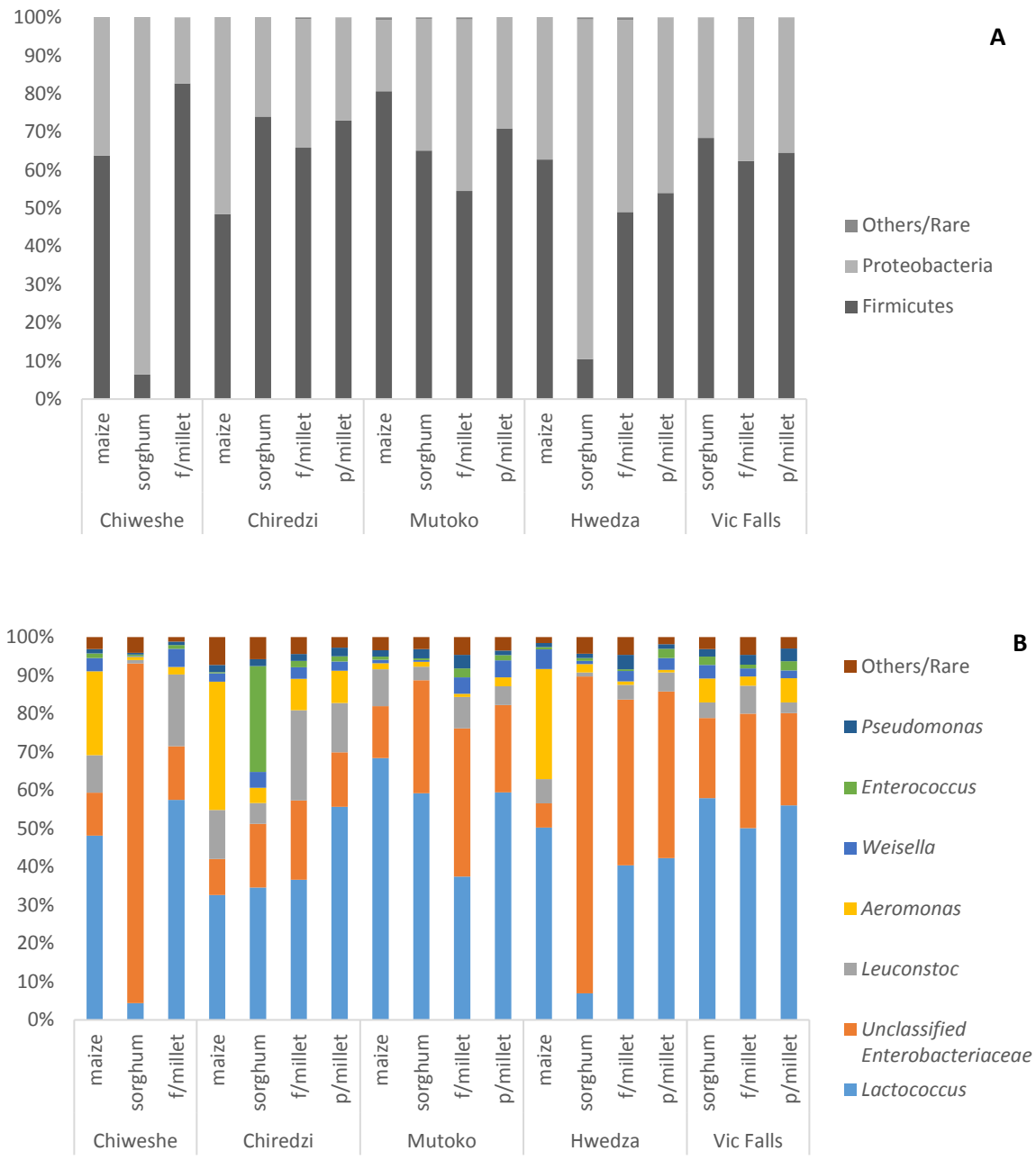

Figure 5.5: Relative abundance of bacterial communities of laboratory fermented slurries at phylum (A) and genus level (B)

f/millet: finger millet, p/millet: pearl millet, sorghum from Chiweshe and Hwedza is red variety while the rest are white varieties, finger millet from Hwedza is white variety while the rest are red varieties. Others/rare represent all phyla or genera with relative abundance of < 1%.

5.4.5 Comparison of bacterial communities of fermented slurries

Among the household finger millet fermented slurries, there was no significant difference between spontaneously fermented slurries and backslopped fermented slurries of the same variety. The bacterial communities of household fermented finger millet slurries comprised of generally the same bacterial consortia, which slightly differed in their relative abundances. The spontaneously fermented white finger millet from the household fermentation were compared with the laboratory fermented. Differences were observed in the relative abundances of unclassified *Enterobacteriaceae* and *Pseudomonas* which were both higher in the laboratory fermented slurries compared to the household fermented slurries ($q < 0.05$).

Pertaining to the laboratory fermented slurries originating from 5 different locations, there were significant differences among the genera *Tolumonas*, *Plesiomonas* and unclassified *Aeromonadaceae* ($q < 0.05$). These potentially pathogenic bacteria were highest in the fermented slurries from Chiredzi compared with other locations. Indeed, these genera were also more abundant in the water from Chiredzi than from other locations. However, there were no differences in dominant and subdominant genera based on location. Based on the type of cereal, significant differences were observed in the relative abundances of unclassified *Enterobacteriaceae*, *Lactococcus*, *Leuconostoc* and *Pantoea* ($q < 0.05$). In all these cases, a distinct difference was observed between red sorghum and maize whereby red sorghum had the highest abundance of unclassified *Enterobacteriaceae* and the lowest abundance of *Lactococcus,* whereas maize had the lowest abundance of unclassified *Enterobacteriaceae* and the highest abundance of *Lactococcus*. *Leuconostoc* abundance was also lowest in red sorghum and highest in maize and red finger millet.

In fact, red sorghum had the highest abundance of Proteobacteria and the lowest abundance of Firmicutes, which is reflected in the PCoA plot (**Figure 5.6**) where the red sorghum is distinguished from other fermented cereals. The PCoA plot shows three distinct groups. Group A shows the water samples from 3 locations except the water from Victoria falls which grouped with the fermented slurries as it had almost 50% *Proteobacteria* and 50% *Firmicutes*. Group B is the red sorghum from Hwedza and Chiweshe and group C is that of the fermented slurries, from both household and laboratory fermentation. Fermented slurries which are more different from each other are those of maize and red sorghum which are positioned on the extremes of axis 1.

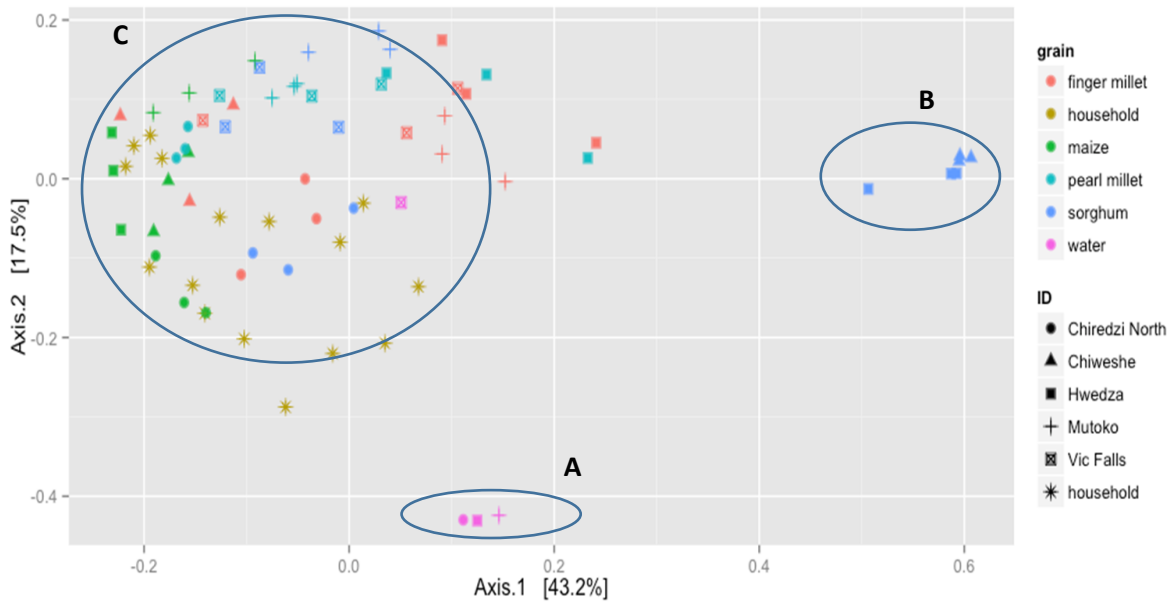

Figure 5.6: Principal Coordinate Analysis (PCoA) based on weighted UniFrac analysis of 16S rRNA gene sequences found in household finger millet fermented slurries, laboratory fermented slurries originating from different locations and water from the respective locations

5.5 Discussion

African cereal fermented slurries are important in the diet of many population groups such that an understanding of their microbial ecosystem is crucial to their improvement. Two studies were carried out; one involving household fermented finger millet slurries and another involving laboratory fermented slurries using different types of cereals typically used in Zimbabwe for the production of porridges. In addition, the preparation of the laboratory fermented slurries was conducted in a way that mimicked the production of fermented slurries from different locations by using the raw materials from the respective locations but controlling the fermentation conditions.

There was no statistical difference between microbiota of spontaneously fermented slurries and backslopped slurries in the households examined. Although a higher level of contaminating or undesirable microbiota in the household fermented slurries compared to laboratory fermented slurries could be expected (Minervini et al., 2015), their microbiota compositions were not significantly different either. The minor differences observed may have been caused by minor differences in fermentation environment and slight variations in the fermentation conditions. However, the present comparison might simply lack power due to the limited number of samples examined. The differences in microbiota composition of fermented slurries from different locations were attributed to rare genera only, which is consistent with studies that failed to show a region specificity in the microbiota composition of sourdoughs (Gobbetti et al., 2016; Van Kerrebroeck et al., 2017). The absence of

specific location microbiota suggests that other parameters in particular the type of raw materials used were the most important drivers of the composition of the cereal fermentation. Alternatively, the present comparison might again lack power.

In both household and laboratory fermented slurries, the dominant and subdominant microbiota generally consisted of *Lactococcus*, unclassified *Enterobacteriaceae*, *Weissella*, *Leuconostoc*, *Aeromonas*, *Enterococcus* and *Pseudomonas* (Figure 5.3 and 5.5). Although relative abundances of these genera varied among the different cereal flour fermentations, the bacterial community at the end of these fermentations was fairly simple. Out of 13 dominant and subdominant genera detected in water samples, only seven were detected in the fermented slurries, and were mostly Firmicutes which, compared to the water samples, increased in abundance and Proteobacteria which generally decreased in abundance. Of the latter, only the unclassified *Enterobacteriaceae* increased in abundance in the fermented slurries. Bacteria associated with cereal flours such as *Acinetobacter*, *Pantoea*, *Comamonas*, *Enterobacter*, *Erwinia* and *Sphingomonas* (Ercolini et al., 2013) were all detected but constituted minor fractions. Although the raw materials used in cereal fermentations may harbor a rich diversity of microbiota, cereal fermentations are often characterized by a succession of dominant and subdominant LAB (Gobbetti et al., 2016). This LAB succession involves the suppression of Gram-negative bacteria and the eventual dominance of lactobacilli. In addition, such microbial ecosystems become less complex due to the adaptation of a limited number of species to the microbial ecosystem (Minervini et al., 2015; Van Kerrebroeck et al., 2017).

The presence of *Lactococcus, Enterococcus* and *Leuconostoc* in the present study is in tandem with the early stages of sourdough fermentation (Figure 5.3 and 5.5). These LAB are cereal endophytes associated with the outer layers of the cereal kernel, and do not tolerate long term acidification (Corsetti et al., 2007; Gobbetti et al., 2016; Minervini et al., 2015; Weckx, Van der Meulen, Allemeersch, et al., 2010). In addition, these organisms were also present in the water implying that the water used from the five locations studied was likely a rich inoculum of these LAB. The genera *Weissella* and *Leuconostoc* were ubiquitous in both household and laboratory fermented slurries. They are indigenous to plant material, widely associated with liquid sourdoughs, and prevail in sourdoughs with a pH above 4 and fermented at low temperatures of < 30^{o}C ,which is characteristic of the fermentation process in the present study (Ampe et al., 1999; Van Kerrebroeck et al., 2017). However, the strong dominance of *Lactococcus* in the present study was unexpected. This organism comprises only a minor part of the dominant LAB in sourdough fermentation (De Vuyst et al., 2014; Nout and Rombouts, 1992) and among African cereal fermentations, only one study indicated the prevalence of *Lactococcus lactis* throughout a 54 h fermentation of *ting* where it was considered well adapted to the sorghum environment (Madoroba et al., 2011). *Lactococcus* has been infrequently observed during

the fermentation of *obushera,* sorghum/millet based gruels (Mukisa et al., 2012; Muyanja et al., 2003), *ben-saalga,* pearl millet slurries (Humblot and Guyot, 2009), maize and sorghum *ogi* and *kunu zaki* (Oguntoyinbo et al., 2011*)* and in some rye, wheat and spelt sourdoughs (Ercolini et al., 2013; Weckx, Van der Meulen, Allemeersch, et al., 2010; Weckx, Van der Meulen, Maes, et al., 2010). The presence of *Lactococcus* as one of the dominant genera in the water samples may also have contributed to its competitive advantage over other LAB (Figure 5.4).

The water samples also harbored a high abundance of unclassified *Enterobacteriaceae* which persisted at the end of the fermentation. Other Proteobacteria which were abundant in water such as unclassified *Comamonadaceae* and *Acinetobacter* were detected in the fermented slurries but in minor fractions, suggesting a potential competitive advantage of *Enterobacteriaceae* over other Proteobacteria. *Enterobacteriaceae* have been found to persist in sourdoughs because of their ability to metabolize acids and tolerate acidic stress (Ercolini et al., 2013; Gobbetti et al., 2016). In wheat sourdough, *Enterobacteriaceae* only started disappearing after 5 days of propagation coinciding with the formation of the mature sourdough (Ercolini et al., 2013). *Enterobacteriaceae* have also been observed in pearl millet slurries sampled from traditional production units of Burkina Faso (Humblot and Guyot, 2009), Mexican maize *pozol* (Ampe et al., 1999), *obushera* (Mukisa et al., 2012), *doklu* (Assohoun-Djeni et al., 2016), *poto poto* (Abriouel et al., 2006) and in Portuguese *broa* made from maize and rye flour (Rocha and Malcata, 2012).

Pertaining to cereal flour type, a major difference was observed between fermented slurries prepared using red sorghum flour and those prepared using the other cereal flours i.e. maize, white and red finger millet, pearl millet and white sorghum (Figure 5.6). *Enterobacteriaceae* constituted 83-89% of the total microbiota of the red sorghum slurries in contrast to 6-54% in other cereal fermented slurries (Figure 5.5). Fermentation of flours from whole cereal grains may result in differences in microbial communities given the presence of dietary fibre and bioactive compounds in the bran fraction (Katina et al., 2012; Katina et al., 2007). More specifically, certain types of phenolic compounds have antibacterial properties that may select for certain bacteria. Maize, sorghum and millets all contain different levels and types of phenolic compounds (Dykes and Rooney, 2006; Gabaza et al., 2018). Among the vast array of phenolic compounds contained in these cereals, red sorghum additionally contains condensed tannins which are not present in the other cereals examined in the present study (Gabaza et al., 2018). Condensed tannins can suppress growth of other bacteria purportedly through their ability to complex with polymers. Low concentrations of 0.2-2% can have a significant effect on the diversity of bacterial populations of an ecosystem (Smith et al., 2005). The fecal microbiota of rats shifted from the predominant bacterial species towards species of *Enterobacteriaceae* and

Bacteroidetes after rats were fed a high condensed tannin diet and the prevailing species were considered to be “tannin resistant” (Smith and Mackie, 2004).

In addition, the antimicrobial activity of red sorghum whole grains was 100-200 times higher than that of the white sorghum and prevented the growth of *Lactobacillus sanfranciscensis* isolated from wheat sourdough (Sekwati-Monang et al., 2012). Only strains of *Lactobacillus casei* and *Lactobacillus parabuchneri* isolated from the sorghum sourdough could grow on the phenolic extracts because of their ability to metabolize phenolic compounds (Sekwati-Monang et al., 2012; Svensson et al., 2010). *L. buchneri* and *L.casei* were isolated from red sorghum that had been fermented for 2-3 days (Sekwati-Monang and Gänzle, 2011) and since the laboratory fermentation in the present study was done for 26 h, lactobacilli with the ability to metabolize phenolic compounds may not had started to proliferate. The use of autochthonous starter cultures with fast acidification properties may thus be important in the case of red sorghum or a longer fermentation may be required to allow the growth of lactobacilli which can metabolize red sorghum phenolic compounds. Pertaining to other cereals i.e. maize, finger millet and pearl millet, perhaps clear differences in their microbial diversity and the influence of flour type in these cereals will be more apparent at the species level.

Although *Enterobacteriaceae* may in fact have a positive functional role in the fermentation, their dominance is a cause of concern as opportunistic pathogenic genera such as *Shigella*, *Salmonella* and *Escherichia* belong to this family. *Aeromonas* and *Pseudomonas* still persisted at end of the fermentation indicating another potential hazard associated with consumption of these products. The fermented cereal slurries will undergo a final cooking process to prepare the porridge and this will eliminate most pathogens but some *Aeromonas* and *Pseudomonas* species can produce toxins that can still render the porridge unsafe. Other cereal fermentations, particularly the non-alcoholic beverages such as *mahewu* from Southern Africa and *obushera* from Uganda, harbor the same potentially pathogenic microbial communities but are consumed without a further cooking step making them high risk products. These products are also used as weaning and complementary foods and some of the detected microorganisms could cause diarrhea in infants (Mukisa et al., 2012).

5.6 Conclusion

Amplicon sequencing of the V4 region of 16S rRNA gene showed the dominance of *Lactococcus* in Zimbabwean maize, sorghum and millet fermented slurries after a fermentation that lasted for 24-36 h. Other LAB commonly associated with the cereal fermentation included *Weissella*, *Leuconostoc* and *Enterococcus* along with some Proteobacteria, Bacteroidetes and Actinobacteria that are typically associated with the raw materials and environment. Large differences in the bacterial communities of

red sorghum compared with other cereal fermented slurries were observed probably because of the presence of condensed tannins in sorghum which may select for the growth of “tannin resistant” bacteria. Fermented slurries could not be differentiated based on origin nor on whether slurries were fermented at laboratory or household level. Although the sequencing in this study did not allow the detection of species-specific composition of the bacterial communities, it led to a deeper understanding of the bacterial composition underlying Zimbabwean maize, sorghum and millet fermented slurries. Yeasts communities have also been observed in African cereal fermented foods so future studies should also consider the use of modern day sequencing techniques to describe the yeasts microbial consortia in order to have a complete view of the microbial ecosystem of African cereal fermented foods.

Chapter 6

Enzymatic degradation of mineral binders in cereals: impact on iron and zinc bioaccessibility

Chapter 6 : Enzymatic degradation of mineral binders in cereals: impact on iron and zinc bioaccessibility

6.1 Abstract

Successful strategies to improve iron and zinc bioaccessibility of cereals can only be realized based on a thorough understanding of the mineral to mineral inhibitor interactions. To this end, we used exogenous enzymes i.e. phytase and a cocktail of phytase, laccase and tannase (P+L+T) to degrade phytic acid and phenolic compounds in order to understand the magnitude of their effects on iron and zinc bioaccessibility of cereals commonly consumed in Africa. Bioaccessibility was defined as the proportion of minerals able to pass through a dialysis membrane of molecular weight cut-off 12-14 kDa while total soluble minerals were the total sum of minerals which were soluble but not dialyzable and that of the dialyzable or bioaccessible fraction. Phytase treatment caused an increase in total soluble zinc from 20.2-59.4 to 29.5-67.6% while an inconsistent effect was observed after treatment with P+L+T. A positive effect on the total soluble iron from 23.9-65.5 to 48.7-87.3% was only observed after treatment of cereals with P+L+T. However, the bioaccessibility of iron and zinc was reduced after both phytase and P+L+T treatments possibly because of interactions between the minerals and the exogenous enzymes. Nevertheless, phytic acid and phenolic compounds have an effect on iron and zinc bioaccessibility and depending on the type of cereal, dietary fibers may also be important.

Keywords : iron, zinc, bioaccessibility, phytase, laccase, tannase

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6.2 Introduction

Iron and zinc deficiencies are global nutritional problems affecting billions of people most of whom reside in developing countries (Gregory et al., 2017). The greatest concern pertaining to iron and zinc deficiencies in low income countries is the low bioavailability of iron and zinc in cereal staples (Gibson et al., 2010). The bioavailability of iron and zinc is modulated by the level of mineral binders in particular, phytic acid (PA), phenolic compounds (PC) including condensed tannins (CT) and mineral absorption enhancers such as ascorbic acid, small organic acids and meat (Gibson et al., 1998). Cereals commonly consumed in Africa i.e. maize, sorghum and millets have high levels of mineral binders and low levels of mineral enhancers and the result of this disproportionate content of mineral binders and enhancers is a low bioavailability of iron and zinc. Several processing methods such as soaking, germination, fermentation and cooking can reduce mineral binders to some extent but they have not been sufficient to improve the mineral bioavailability substantially (Baye et al., 2014; Gabaza et al., 2017b; Hotz and Gibson, 2007).

The utilization of exogenous enzymes targeting mineral binders in cereals may provide some important insights on their relative effects on iron and zinc bioavailability. The use of exogenous phytase to completely degrade PA in cereals increased the absorption of iron in healthy men and women by different magnitudes (Hurrell et al., 2003). The highest increase in absorption was observed for wheat (12 fold), maize and oats (5-8 fold), rice (3-5 fold), and low tannin sorghum (2 fold). On the other hand, there was no increase in absorption for the high tannin sorghum. The lack of an increase in the high tannin sorghum was attributed to the inhibitory effect of PC and CT which are even more potent inhibitors than PA. In one *in vitro* study, Baye et al. (2015) showed that dephytinization of a flour blend from wheat-red sorghum and tef-white sorghum used to make *injera*, did not increase the bioaccessibility of iron but only increased iron bioaccessibility after using a combination of PC degrading enzymes (polyphenol oxidase) and cell wall degrading enzymes (cellulase and xylanase) in addition to the phytase treatment. This infers that in these type of cereals, dephytinization is not adequate as PC and dietary fibers also play a critical role in modulating mineral bioavailability. The need for phytase in combination with either PC or cell wall degrading enzymes to improve mineral bioavailability of sorghum and millets has been demonstrated (Lestienne et al., 2005b; Matuschek et al., 2001).

The addition of phytase to pearl millet bran only increased iron and zinc solubility when phytase was combined with xylanase (Lestienne et al., 2005b). Dephytinization of high fiber cereal flours from whole cereal grains may probably be insufficient to improve mineral bioavailability of cereals and since the study of Hurrell et al. (2003) was conducted on decorticated cereals, the effect of dietary fibers or

their complexation with mineral binders was not apparent. Secondly, the variation in the magnitude of response after dephytinization indicates a huge matrix effect, signifying that strategies to improve mineral bioavailability of cereals may need to be customized for different cereal types. The localization of minerals and mineral binders coupled with the differential mineral binding throughout the different tissue fraction of cereals (Eagling et al., 2014) elicits differences in response to processes.

The objective of this study was thus to gain insight on the effect of exogenous enzymes on the bioaccessibility of iron and zinc of different whole cereal grains commonly consumed in Africa i.e. maize, red and white sorghum, pearl millet and red and white finger millet. The enzymes evaluated in this study were phytase for the degradation of PA, and laccase and tannase for the degradation of PC and CT respectively. An increased understanding of the interaction between the inhibitors and minerals in different cereal matrices is important for the formulation of strategies that can better target the mineral binders.

6.3 Materials and methods

6.3.1 Materials

Maize, red sorghum, pearl millet and red and white finger millet were obtained from farmers of the Hwedza Communal area while white sorghum was obtained from Mutoko, Zimbabwe. Wheat 3-phytase enzyme was kindly donated by Nuscience (Drongen, Belgium). ICP multi-element standard solution IV was purchased from Merck (Germany) and all other reagents and materials were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA) i.e. phytic acid dodecasodium salt, 2,2'-bipyridine and thioglycolic acid, Folin Ciocalteu's reagent, gallic acid, vanillin, catechin, tannase from *Aspergillus ficuum* (150 units/g), laccase from *Trametes versicolor* (10 units/mg), α-amylase from porcine pancreas (10 units/mg solid), pepsin from porcine gastric mucosa (3200-4500 units/mg protein), pancreatin from porcine pancreas (8xUSP), dialysis bags (MW cut off 12-14 kDa) and bile from porcine bile extract.

6.3.2 Methods

6.3.2.1 Enzyme treatment of cereals

The cereal grains were carefully cleaned to remove all foreign particles and were milled using a laboratory hammer mill equipped with sieve of size 0.5 mm. To prevent fermentation during the enzymatic incubation, the flours were dry heat sterilized at 160^{o}C for 6 min. Dry heat sterilization at this time and temperature combination does not cause significant changes on starch structures and their digestibility (Yue et al., 1999), nor on mineral binders (Baye et al., 2015). Enzyme treatment was

done by mixing flour and 0.1 M sodium acetate buffer (pH 4.5) in a 1:3 ratio (w/v). Two enzymatic treatments were done i.e. phytase treatment (30 U/g) and another treatment of phytase (30 U/g) + laccase (6 U/g) + tannase (1 U/g) treatment (P+L+T). The concentrations of the enzymes used and the duration of incubation to obtain maximal degradation of PA, PC and CT were determined during preliminary experiments. The enzyme flour mixtures were incubated at 30^{o}C on a shaker for 2 h for the phytase treated cereals and 4 h for the P+L+T treated cereals. After enzyme treatment, the enzymes were deactivated by heating for 1 min in a boiling water bath. The enzyme treated flours were then frozen and lyophilized. Three sets of each cereal flours, i.e. non-enzyme treated (NET – partly described in chapter 5 as cereal flours originating from Hwedza and white sorghum from Mutoko), phytase treated, P+L+T treated cereals were then analyzed for mineral binders, iron and zinc contents and iron and zinc bioaccessibility.

6.3.2.2 Analysis

The dry matter content of the flours, iron and zinc contents, mineral binders and iron and zinc bioaccessibility were analyzed according to the protocols described in section 3.3.2.3-3.3.2.11. Bioaccessibility was defined as the proportion of minerals able to through a dialysis membrane of 12-14 kDa molecular weight cut-off.

6.3.2.3 Statistical Analysis

Data were subjected to two-way ANOVA to check for the combined effect of enzyme treatment and type of cereal. There was significant interaction in all cases hence simple effects were considered. Tukey's post-hoc test was employed to decipher where differences existed ($p < 0.05$). Analysis was carried out using IBM SPSS software version 23. Data is shown as means ± standard deviations of three independent samples.

6.4 Results

6.4.1 Content of mineral binders

The level of mineral binders was determined in phytase and P+L+T treated cereals to determine the effectiveness of the enzyme treatment to reduce mineral binders. **Table 6.1** shows the results of the soluble and bound PC and CT of the cereals. Phytase treatment caused variable responses to the soluble PC with increases observed for pearl millet, red and white finger millet while a decrease was observed for red sorghum and no change in maize and white sorghum. With respect to the P+L+T

treated cereals, soluble PC was reduced for all cereals except for pearl millet where there was no change observed. Treatment with phytase caused a decrease of 16-35% in the bound PC of maize, white sorghum, red and white finger millet and no change was obtained for red sorghum and pearl millet. On the other hand, treatment with P+L+T elicited a decrease only for maize and red finger millet while no change was generally observed for the other cereals. CT were only detected in red sorghum and red finger millet of NET cereals. Phytase treatment caused an increase in the CT content of both red sorghum and red finger millet while treatment with P+L+T caused a high decrease of 75% in red sorghum and 69% in red finger millet. Overall, there was no change in the total PC of red sorghum and pearl millet after phytase treatment since the bound PC fraction which constituted more than 75% of the total PC was not affected by the treatment. On the other hand, the total PC of maize, white sorghum, red and white finger millet was reduced by 13-32% after phytase treatment. In terms of the P+L+T treatment, a reduction of 17% and 27% was observed for maize and red finger millet respectively, while no change was observed for the rest of the cereals.

Both phytase and P+L+T treatment completely degraded PA of maize, red and white sorghum and pearl millet **(Table 6.2)**. On the other hand, PA of red and white finger millet was not completely degraded after both types of enzymatic treatments with reductions of 71 to 84% for red finger millet and 55 to 65% for white finger millet.

Table 6.1: Content of soluble, bound phenolic compounds and total condensed tannins of whole grain cereal flours

	NET	Phytase	P+L+T	P value
		Soluble PC		
Maize	$50.6\pm0.7^{b,A}$	$45.5\pm4.9^{b,A}$	$20.0\pm0.6^{a,B}$	<0.001
Red sorghum	$798\pm20^{c,C}$	$667\pm25^{b,C}$	$156\pm9^{a,D}$	<0.001
White sorghum	$37.7\pm3.1^{b,A}$	$44.4\pm3.1^{b,A}$	$21.9\pm1.9^{a,B}$	<0.001
Pearl millet	$77.9\pm4.1^{a,B}$	$112\pm5^{b,B}$	$72.0\pm2.5^{a,C}$	<0.001
Red finger millet	$78.2\pm3.1^{b,B}$	$104\pm1^{c,B}$	$17.8\pm0.8^{a,B}$	<0.001
White finger millet	$30.1\pm1.6^{b,A}$	$35.0\pm0.9^{c,A}$	$8.49\pm0.41^{a,A}$	<0.001
P value	<0.001	<0.001	<0.001	
		Bound PC		
Maize	$580\pm15^{c,B,C}$	$385\pm8^{a,A}$	$440\pm35^{a,A}$	<0.001
Red sorghum	1410 ± 113^{D}	1541 ± 131^{C}	1709 ± 89^{C}	0.077
White sorghum	$422\pm16^{b,A}$	$356\pm31^{a,A}$	$475\pm8.5^{c,A}$	0.001
Pearl millet	597 ± 33^{C}	473 ± 93^{A}	540 ± 34^{A}	0.154
Red finger millet	$1388\pm55^{b,D}$	$1057\pm36^{a,B}$	$1200\pm97^{a,B}$	0.003
White finger millet	$438\pm26^{b,A,B}$	$286\pm22^{a,A}$	$434\pm29^{b,A}$	0.001
P value	<0.001	<0.001	<0.001	
		CT		
Red sorghum	$524\pm60^{b,B}$	$856\pm36^{c,B}$	$130\pm65^{a,B}$	<0.001
Red finger millet	$43.8\pm3.3^{a,A}$	$159\pm16^{c,A}$	$13.5\pm4.7^{b,A}$	<0.001
P value	<0.001	<0.001	<0.001	

NET: non enzyme treated, P+L+T: phytase+laccase+tannase, PC: phenolic compounds, CT: condensed tannins. Values with different capital superscript letters within columns are significantly different. Values for soluble and bound PC are expressed in mg GAE/100 g dm, while values for CT are expressed in mg CE/100 g dm. Values with different small superscript letters across rows are significantly different, $p < 0.05$, n=3. GAE: gallic acid equivalents, CE: catechin equivalents.

Table 6.2: Phytic acid content of whole grain cereal flours

	NET	Phytase	P+L+T	P value
Maize	2197±88^{D}	ND	ND	<0.001
Red sorghum	703±17^{A}	ND	ND	<0.001
White sorghum	1648±246^{C}	ND	ND	<0.001
Pearl millet	1232±46^{B}	ND	ND	<0.001
Red finger millet	1153±28^{c,B}	189±37^{a,B}	334±35^{b,B}	<0.001
White finger millet	1043±39^{c,B}	365±95^{a,C}	466±14^{b,C}	<0.001
P value	<0.001	<0.001	<0.001	

NET: non enzyme treated, P+L+T: phytase+laccase+tannase, ND: not detected. Values with different capital superscript letters within columns are significantly different. Values are expressed in mg/100 g dm. Values with different small superscript letters across rows are significantly different, $p < 0.05$, n=3, limit of detection for PA was 9.09 µg/ml.

6.4.2 Iron and zinc contents

Figure 6.1 shows the iron and zinc contents of the cereals. The iron content ranged from 2.97 to 10.3 mg/100 g dm with lower iron content observed for maize, white finger millet and red finger millet while higher contents were observed for white sorghum, red sorghum and pearl millet. The zinc contents ranged from 1.17 to 3.71 mg/100 g dm and were lowest for both red and white finger millet and highest for pearl millet. Maize contained almost equal levels of iron and zinc while for the rest of the cereals, the iron contents were 3-4 times higher than that of zinc. Iron and zinc contents of the phytase and P+L+T treated cereals were not determined as no significant change in their levels was expected.

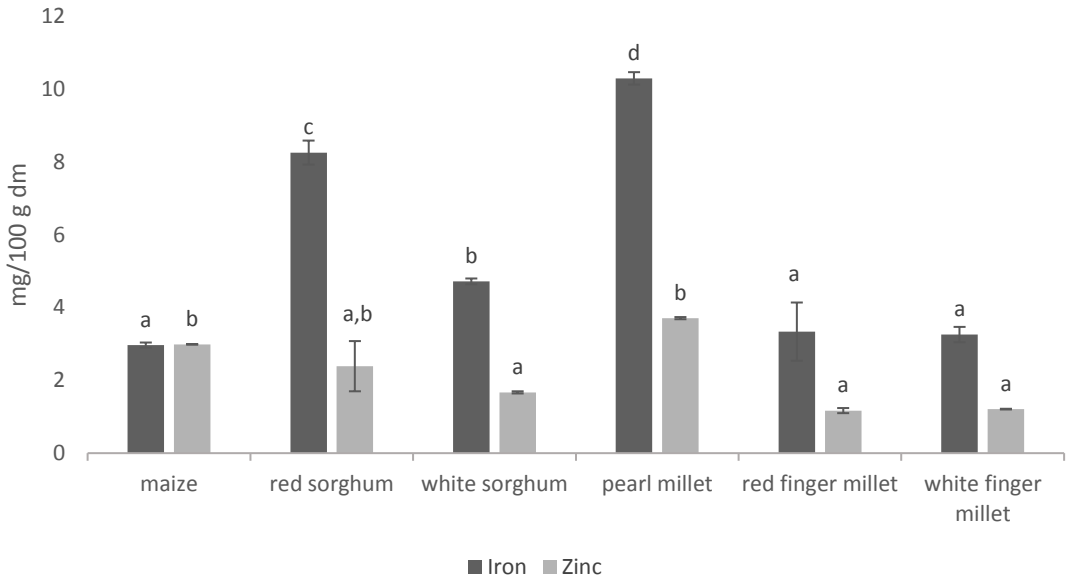

Figure 6.1: Iron and zinc contents of whole cereal grain flours

Bars of the same color with different small letters are significantly different, $p < 0.05$, n=3.

6.4.3 Iron and zinc bioaccessibility

The total soluble iron (%SND+%D) of the NET cereals was 22.9±0.95% for red sorghum, 43.6±2.89% for white sorghum, 47.1±3.48% for pearl millet, 65.5±10.4% for maize, 58.8±0.76% for red finger millet and 62.8±4.77% for white finger millet. An increase in the total soluble iron was observed for red and white sorghum treated with phytase while no change was observed for the other cereals. Pertaining to P+L+T cereals, an increase in the total soluble iron was observed in all cereals compared to the NET cereals. However, comparing the phytase and P+L+T cereals, no change in the total soluble iron was observed for red and white sorghum while an increase was generally observed for the rest of the cereals.

The %SND iron of the NET cereals ranged from 22.7-46.3% with the lowest %SND observed for red and white sorghum and highest for maize. On the other hand, comparable %SND iron was observed for red and white finger millet and pearl millet **(Figure 6.2A)**. No effect in phytase treatment was found for maize, red sorghum, pearl millet and red finger millet while an increase in %SND iron was found for white finger millet and white sorghum. With respect to the treatment with P+L+T, an increase in the %SND iron was observed in all cereals with the highest increase found for maize. Comparing the phytase and P+L+T cereals, no change was observed for white sorghum and finger millet.

Concerning the bioaccessibility of iron estimated by the proportion of iron which was able to pass through a dialysis membrane of 12-14 kDa molecular weight cut-off, it ranged between 1.23-36.7%

with the lowest observed for red sorghum and highest observed for white finger millet **(Table 6.3)**. Phytase treatment caused no change in the iron bioaccessibility of maize, pearl millet and red and white finger millet while a decrease was found for white sorghum and an increase for red sorghum. The treatment with P+L+T did not elicit any change in the iron bioaccessibility of pearl millet and white finger millet whereas it caused a decrease in the iron bioaccessibility of maize, red finger millet and white sorghum and an increase in red sorghum. The iron bioaccessibility of phytase treated cereals of red sorghum and red finger millet were higher than the P+L+T treated counterparts while for the other cereals, there was no difference in iron bioaccessibility between the phytase and the P+L+T cereals.

Concerning zinc, the total soluble zinc of NET cereals ranged between 20.2-59.4% with the highest total soluble zinc observed for pearl millet and lowest for white and red sorghum. Phytase treatment caused no change in the total soluble zinc of pearl millet while an increase was observed for the rest of the cereals. With respect to the P+L+T cereals, no change was observed for red sorghum and white finger millet while an increase was observed for maize, pearl millet, white sorghum and red finger millet. Comparing phytase and P+L+T cereals, higher %total soluble zinc was observed for phytase treated cereals of red sorghum, red finger millet and white finger millet while higher %total soluble zinc was observed for P+L+T cereals of pearl millet and white sorghum.

The %SND zinc of NET was between 11.1-47.7 again with highest %SND for pearl millet and comparable %SND zinc for the rest of the cereals **(Figure 6.2B).** In general, an increase in the %SND zinc was observed after phytase treatment in all cereals except for pearl millet. An increase was also observed in the %SND zinc of P+L+T cereals of maize, white sorghum, red and white finger millet. No effect was observed in the P+L+T treatment of maize, pearl millet and red finger millet compared to their phytase treated counterparts. The zinc bioaccessibility of the NET cereals ranged from 8.38-21.4% being lowest for red and white sorghum and not significantly different for the rest of the cereals **(Table 6.3)**. The phytase treatment caused either no change (red sorghum, pearl millet, red finger millet) or a decrease in the zinc bioaccessibility (maize, white sorghum, white finger millet). Likewise, the P+L+T treatment also had mostly no effect on the zinc bioaccessibility except for maize and white finger millet where there was a decrease. There were generally no differences between the phytase and P+L+T zinc bioaccessibility of the cereals.

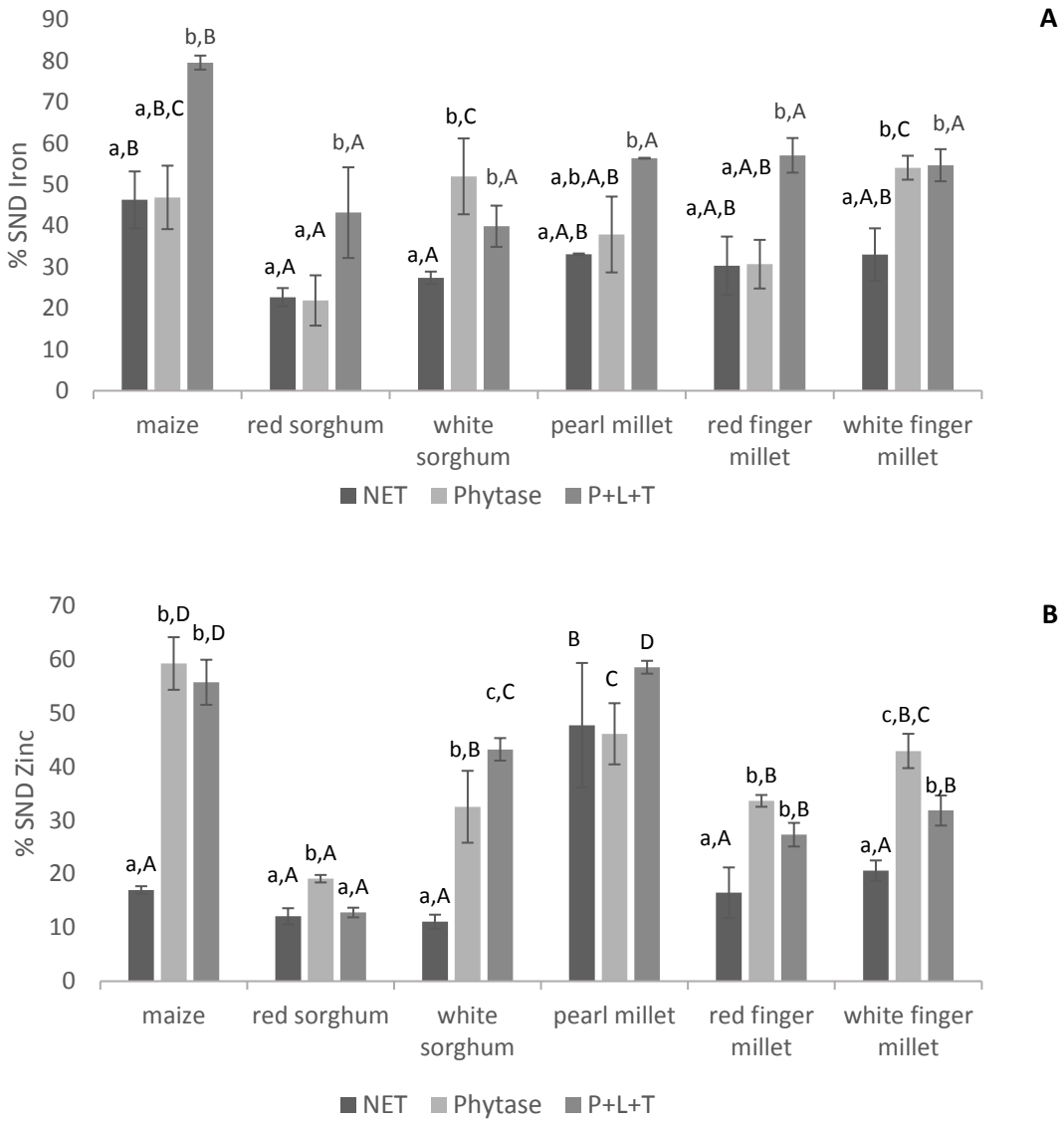

Figure 6.2: Soluble non dialyzable iron (A) and zinc (B) of whole grain cereal flours

NET: non enzyme treated, P+L+T: phytase+laccase+tannase, SND: soluble non dialyzable. Bars of the same color with different capital letters are significantly different, and bars of the same cereal grain with different small letters are significantly different $p < 0.05$, n=3.

Table 6.3: Bioaccessible iron and zinc (%) of enzyme treated whole grain cereal flours

	NET	Phytase	P+L+T	P values
Bioaccessible (dialyzable) iron				
Maize	19.3±3.5^{b,B}	14.0±2.0^{a,b,A}	7.71±0.63^{a}	0.036
Red sorghum	1.23±0.29^{a,A}	28.8±2.5^{c,B}	10.7±2.0^{b}	<0.001
White sorghum	16.2±1.4^{b,B}	9.36±1.39^{a,A}	8.89±1.83^{a}	0.013
Pearl millet	14.1±3.3^{B}	8.79±1.74^{A}	9.22±0.05	0.151
Red finger millet	32.6±8.8^{b,B}	31.0±7.3^{b,B}	9.74±5.88^{a}	0.045
White finger millet	36.7±12.6^{B}	14.3±1.3^{A}	12.8±3.4	0.070
P value	0.010	<0.001	0.649	
Bioaccessible (dialyzable) zinc				
Maize	17.6±4.2^{b,A,B}	7.79±3.08^{a,A,B}	9.32±1.16^{a,A,B,C}	0.033
Red sorghum	9.21±5.23^{A}	10.6±1.3^{B,C}	5.28±0.17^{A}	0.305
White sorghum	8.38±0.60^{b,A}	3.74±1.15^{a,A}	6.43±1.18^{a,b,A,B}	0.020
Pearl millet	11.7±1.3^{A,B}	13.8±2.4^{B,C}	12.8±0.1^{C}	0.518
Red finger millet	13.2±2.0^{A,B}	13.1±1.2^{B,C}	11.3±3.2^{A,B,C}	0.615
White finger millet	21.4±1.5^{b,B}	14.1±3.1^{a,C}	12.1±0.9^{a,B,C}	0.010
P value	0.009	<0.001	0.011	

NET: non enzyme treated, P+L+T: phytase+laccase+tannase. Values with different capital superscript letters within columns are significantly different. Values with different small superscript letters across rows are significantly different, $p < 0.050$, n=3.

6.5 Discussion

Solubility of minerals at the physiological pH of the intestine is a key prerequisite for their absorption. An increase in the total soluble minerals is thus a positive indication of the capacity of a process to release bound minerals. The total soluble iron and subsequent bioaccessibility of the NET cereals was lowest for red sorghum and highest for white and red finger millet (Table 6.3). Hurrell et al. (2003) also reported low iron bioaccessibility of red sorghum due to the presence of CT which are considered to be more inhibitory than PA. The inhibitory effect of CT was not apparent in red finger millet since both red and white finger millet had the highest and comparable iron bioaccessibility even though the red variety contained CT. The CT in finger millet are mainly oligomers of catechin (Chandrasekara and Shahidi, 2011; Gabaza et al., 2016) and are probably not strong mineral binders compared to red sorghum CT. The study by Platel et al. (2010) also reported comparable iron bioaccessibility between

white and brown finger millet varieties. Red sorghum also had the lowest zinc bioaccessibility indicating that the potency of red sorghum CT can also be extended to zinc absorption. Generally, phytase did not improve neither the bioaccessibility nor the total soluble iron of most of the cereals. On the other hand, phytase treatment caused an increase in the total soluble zinc of all cereals counteracted by generally no change in the zinc bioaccessibility or a decrease in the case of maize and white sorghum.

There are clear differences in the response of iron and zinc to phytase treatment. It has been suggested that the most potent inhibitor of zinc absorption is PA (Lestienne et al., 2005b; Towo et al., 2006) and this was proven in our study whereby an increased total soluble zinc was observed in all phytase treated cereals. Unlike iron, zinc is evenly distributed in the grain and only about 10% has been found in the bran of pearl millet, 23% in the bran of sorghum (Hama et al., 2011; Lestienne et al., 2007) and up to 20% in the seed coat of finger millet (Krishnan et al., 2012). The distribution of PA in cereal grains varies with most cereal grains having about 90% of the PA in the aleurone layer and about 10% in the scutellum (Brinch-Pedersen et al., 2014). However, this is not the case for some cereal grains such as maize with about 90% of its PA in the scutellum and only 10% in the aleurone layer (Brinch-Pedersen et al., 2014). Pearl millet has about 60% PA located in the germ, 30% in the endosperm and 10% in the bran and for sorghum, up to 15% of PA is in the bran fraction (Hama et al., 2011; Lestienne et al., 2007). If the bulk of the PA is found in one part of the grain, this PA is likely not to be bound to a large proportion of zinc since zinc is evenly distributed throughout the grain. An increase in total soluble zinc after phytase treatment thus entails that a significant proportion of zinc in the cereals studied is existing as PA complexes. For finger millet however, it appears that a large proportion of the PA may be found in the outer layers bonded to dietary fibers because the phytase treatment was not able to completely degrade all the PA. Furthermore, a combination of P+L+T also did not improve PA degradation (Table 6.2). Krishnan et al. (2012) reported that 30% PA and 70% of the total PC are localized in the seed coat of finger millet hence this PA is likely to be strongly bonded to dietary fibers and PC. Phytase treatment could also have increased total soluble zinc because zinc is known to be complexed to PA through heterometal complexes such as calcium-PA-zinc complexes which are very strong and zinc saturated PA complexes which are insoluble (Eagling et al., 2014) thereby requiring phytase to be broken down.

On the contrary, the reason why total soluble iron did not increase in most of the cereals after phytase treatment suggests that a large part of iron is probably complexed with PA via soluble complexes or is complexed with PA together with PC and dietary fibers. Iron is not uniformly distributed in cereal grains and a large part is found in the outer part of the grains. In pearl millet, sorghum and finger millet, up to 41-50% of iron can be found in the bran fraction (Hama et al., 2011; Krishnan et al., 2012). If 50% of

iron is in the bran faction, then the rest of the iron should be found in the inner parts of the grain and should be solubilized by phytase treatment if it is bound to PA. Interestingly, about 50% of the iron of all the cereals except red sorghum was soluble in the NET cereals purporting that if PA is complexed to Fe in the inner parts of the grain, the PA-iron complexes are probably soluble or not strong. There is a paucity of information concerning the speciation of iron in maize, sorghum and millets but recently Eagling et al. (2014) found that about 23-29% of iron in wheat was soluble and bound to complexes of non-protein amino acid nicotianamine, 2'-deoxymugineic acid and PA as monoferric phytate and also some high molecular weight compounds which were not identified. The nicotianamine and 2'-deoxymugineic acid complexes were of low molecular weight (1.5 kDa) while the monoferric phytate was about 5 kDa. This result is corroborated by the finding of Simpson et al. (1981) who found that monoferric phytate was soluble at neutral pH and also potentially bioavailable in humans. Monoferric phytate has been found in several cereal grains such as wheat, rice and maize (Engle-Stone et al., 2005). Although this finding in wheat cannot be extrapolated to other types of grains, we speculate that the soluble iron of our cereals could be bound to these low molecular weight compounds which are potentially bioaccessible.

The generally lower iron and zinc bioaccessibility of the phytase treated cereals could be a result of chelation of the exogenous phytase enzyme with minerals forming high molecular weight soluble complexes (> 14 kDa) that are not dialyzable thus not bioaccessible (Lestienne et al., 2005b). A positive response in iron bioaccessibility after phytase treatment was only observed for red and white sorghum (Table 6.3). This was an unexpected result particularly for red sorghum as dephytinization of high tannin sorghum without removal of PC and CT has been found to be insufficient to improve iron bioaccessibility (Baye et al., 2015; Hurrell et al., 2003; Matuschek et al., 2001; Towo et al., 2006). However, the total soluble iron of sorghum was comparable to that of the other cereals after dephytinization. This phenomenon could be related to the differences in speciation of iron in cereals and their milling fractions further pointing out the gap in information pertaining to the structural chemistry of iron in maize, sorghum and millets.

Baye et al. (2015) showed that the dephytinization of wheat-red sorghum blend and tef-white sorghum blend did not increase neither the total soluble iron nor its bioaccessibility. Similar to our findings, they also observed that treatment of both the tef-white sorghum and wheat-red sorghum blend with PC degrading enzyme increased the soluble iron but did not increase the iron bioaccessibility of the wheat-red sorghum. Close to 90% of the iron in maize was solubilized after P+L+T treatment while for both red and white sorghum, up to 54% of iron was solubilized and about 67% for millets. The increase in soluble iron shows that part of the iron in cereals is perhaps bound to PC and also CT or that the release of soluble compounds with potential to bind iron may contribute to solubilizing part of the insoluble

iron. According to Matuschek et al. (2001) and Towo et al. (2006), simultaneous treatment of sorghum and red finger millet with phytase and PPO increased the amount of soluble iron. The treatment of the flours with PPO alone did not yield an improvement in the soluble iron probably because PC are bonded to PA and/or dietary fibers. Moreover, in all these studies, a large proportion of PC was not reduced by the enzymatic treatment. In our study, we used laccase which has a wide substrate specificity and can oxidize a wide range of phenolic compounds such as mono, di, and polyphenols (Cannatelli and Ragauskas, 2017; Yang et al., 2017) but it was only able to have a huge effect on the soluble PC and not on the bound PC. The bound PC constituted up to 95% of the total PC and if PC are binding iron, then most of the iron is likely to be bound to the bound PC (Table 6.1). Indeed, in all cereals except maize, a significant part of the iron was not solubilized after P+L+T treatment and could thus be associated with bound PC which are mostly bound to cell wall components (Acosta-Estrada et al., 2014). Almost all of the iron in maize was solubilized after the P+L+T treatment suggesting that in this matrix, dietary fibers may have a minimal role in iron binding or the enzymatic treatment was efficient in releasing bound iron. CT in red sorghum were drastically reduced by the P+L+T treatment but their reduction could only solubilize 33.1% of the iron. A probable reason for this is the well-known reaction of CT with macromolecules such as proteins, causing a reduction in their extractability and assayability (Taylor and Duodu, 2015) thus the iron could still be bound in protein-tannin complexes.

In our preliminary enzymatic experiments (data not shown), the incubation of cereals with either laccase or tannase alone did not cause a significant reduction in PC and CT, respectively. However, the combination of P+L+T caused a reduction of soluble PC and CT possibly because the enzymes work in a synergistic manner by disrupting the PA, PC and CT interactions thereby bringing each substrate in close proximity to its enzyme. There was also evidence of liquefaction in the P+L+T treated cereals indicating the occurrence of some depolymerization reactions. Unfortunately, iron bioaccessibility was not improved probably because iron was still probably bound to soluble high molecular weight compounds (> 14 kDa) which were not dialyzable thus not bioaccessible. One of the end products of tannase is gallic acid which binds iron through its galloyl groups (Khokhar and Owusu Apenten, 2003) and this could be one of the constituents of the SND fraction. The exogenous enzymes themselves could also have participated in iron binding as after phytase treatment, 16 to 55% of the soluble iron was bioaccessible while after P+L+T treatment, only 8 to 20% of iron was bioaccessible which may imply that bioaccessibility decreased according to the amount and type of exogenous enzymes added (Table 6.3).

An increased iron bioaccessibility was observed after treatment of cereals with a combination of phytase and cell wall degrading enzymes; cellulase and hemicellulase, showing that dietary fibers may be the missing link to understanding interaction between minerals and mineral binders (Baye et al.,

2015). The effect of dietary fibers on mineral bioaccessibility is highly controversial but the main question is whether dietary fibers act on their own in inhibiting mineral bioaccessibility or if they act as a result of their complexation with PA, PC and CT. A study by Antony and Chandra (1999) showed that the use of cell wall degrading enzymes alone in finger millet did not increase the solubility of iron suggesting that dietary fibers do not inhibit mineral bioaccessibility on their own in finger millet. On the other hand, Baye et al. (2015) purported that the effect of dietary fibers was independent of PA although this is difficult to conclude because the cell wall degrading treatment was done on dephytinized flours where the effect of PA was no longer apparent. Since still some iron remained insoluble after the P+L+T treatment in our study, we can assert that the insoluble iron is probably complexed to bound PC and dietary fibers. A study by Lestienne et al. (2005b) showed that 63% of iron in decorticated pearl millet and 35% in bran was soluble after phytase and xylanase treatment and they attributed the remaining fraction of iron to the effect of PC. Release of iron in whole cereal grains may thus require a combination of PC and cell wall degrading enzymes. The differences in the magnitude of iron solubilized in the different cereals can be attributed to the differences in the structure and composition of their PC which influences their mineral binding ability.

In comparison to the phytase treatment, P+L+T treatment did not induce a positive effect on neither the total soluble zinc nor its bioaccessibility of red sorghum and red finger millet. This observation further proves that zinc in some grains is mostly bound to PA. A combination of phytase and xylanase treatment yielded almost 100% soluble zinc in decorticated pearl millet vs. 69% in the bran fraction (Lestienne et al., 2005b). The remaining zinc was mainly attributed to tannin-zinc and protein-zinc complexes and since we found CT in red sorghum, it is possible that tannin-zinc complexes dominated. A positive effect of the P+L+T treatment on the total soluble zinc was found for white sorghum and pearl millet with no effect on the bioaccessibility. Besides its complexation with PA, zinc is also suggested to be bound to proteins in particular sulphur containing amino acids due to its role in protein synthesis and membrane structure (De Brier et al., 2016; Eagling et al., 2014). Depolymerization reactions that could have occurred during the P+L+T treatment could have necessitated improved protein digestibility allowing release of zinc from protein-zinc complexes. Again, effect of exogenous enzymes could have retarded zinc bioaccessibility.

6.6 Conclusion

The use of exogenous enzymes is crucial for the understanding of mineral and mineral binder interactions. Total soluble iron which was the sum of soluble but not dialyzable iron and dialyzable iron (bioaccessible iron, < 14 kDa) was only improved after treatment of cereals with P+L+T and not after phytase treatment. This suggests that an increase in soluble compounds with ability to bind iron may contribute to solubilize some of the insoluble iron, or that some proportion of iron in these cereals is complexed to PC. On the other hand, the P+L+T treatment did not have an effect on zinc solubility while phytase treatment increased the total soluble zinc of all cereals suggesting that a high proportion of zinc in cereals is most likely bonded to PA. Although total soluble minerals were increased after either phytase or P+L+T treatment, the bioaccessibility (proportion of minerals able to pass through a dialysis membrane of 12-14 kDa molecular weight cut-off) was not improved, probably because of interactions between minerals and exogenous enzymes forming high molecular weight compounds which were not dialyzable. A significant part of the iron and zinc remained insoluble after both enzyme treatments thereby highlighting the existence of other mineral binders. The effect of dietary fibers on both iron and zinc bioaccessibility and their interaction with PA, PC, CT requires further investigation. Furthermore, studies on the speciation of iron and zinc in maize, sorghum and millets are highly needed in order to fully comprehend the mineral and mineral binder interactions in these cereals.

Chapter 7

Baobab fruit pulp and mopane worm as potential functional ingredients to improve the iron and zinc bioaccessibility of fermented cereals

Chapter 7 : Baobab fruit pulp and mopane worm as potential functional ingredients to improve the iron and zinc bioaccessibility of fermented cereals

7.1 Abstract

Cereals are of low iron and zinc bioavailability because of high phytic acid (PA) and phenolic compounds (PC). Affordable and sustainable food based strategies are crucial in alleviating mineral deficiencies and food-to-food fortification is one strategy that is feasible even for the poorest communities. In the present study, we evaluated the effect of adding ascorbic acid rich baobab fruit pulp and the "meat factor" from mopane worm to fermented cereals on iron and zinc bioaccessibility (proportion of dialyzable iron, < 14 kDa). A positive effect in iron and zinc % bioaccessibility was observed for baobab fruit pulp enriched cereals (BEC) while for mopane enriched cereals (MEC), a negative effect was generally observed. However, amounts of bioaccessible iron were comparable or even higher for MEC than for BEC and non-enriched cereals (NEC) indicating that enrichment of cereals with baobab fruit pulp or mopane worm may make a meaningful contribution to iron nutrition. The amount of bioaccessible zinc was low in all enriched cereals, hence strategies to increase the bioaccessibility and level of zinc in cereals are urgently needed.

Keywords: iron, zinc, ascorbic acid, "meat factor", mopane worm, baobab fruit pulp, fermented cereals

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7.2 Introduction

Iron and zinc deficiencies arising from a high consumption of monotonous cereal staples is mainly as a result of mineral binders in particular phytic acid (PA) and phenolic compounds (PC) which are rife in cereals and cereal products (Gibson et al., 2006). The most utilized approaches used to combat iron and zinc deficiencies include fortification, supplementation and biofortification (Gregory et al., 2017; Saini et al., 2016). These strategies have positive effects in many circumstances but in some developing countries like Zimbabwe, the risk of iron and zinc deficiencies still exists owing to the challenges and limitations presented by these strategies in low income settings (Gabaza et al., 2017a).

One approach that has recently emerged is food-to-food fortification using iron and zinc rich food sources and/or food sources such as ascorbic acid rich foods and meat which have the capacity to improve iron and zinc bioavailability (Lung'aho and Glahn, 2009, Cercamondi et al., 2014a). This strategy therefore entails the identification of locally available food sources which are either high in iron and/or zinc or with potential to increase bioavailability. Since these food sources are locally available, they are easily accessible to even the poorest communities hence offering a sustainable approach to prevent mineral deficiencies in vulnerable populations (Kruger et al., 2015).

Several African food sources have been identified to have high iron and zinc contents and these include many indigenous green leafy vegetables such as amaranth leaves, spider plant, jute leaves and pumpkin leaves with iron and zinc contents ranging between 3-109 mg/100 g dm and zinc 11-85 mg/100 g dm, respectively (Odhav et al., 2007). In addition to high iron and zinc contents, certain fruits and vegetables also have a high amount of mineral enhancers and of particular interest is baobab fruit (*Adansonia digitata*) whose pulp contains a high ascorbic acid content ranging between 150-500 mg/100 g dm (Chadare et al., 2008; Parkouda et al., 2012). Furthermore, a variety of edible insects are commonly consumed in many African countries and provide affordable high quality protein comparable to that of animal meat (Verkerk et al., 2007). These insects contain high amount of iron (1.30-1562 mg/100 g) and zinc (4.27-26.5 mg/100 g) and it has been suggested that their consumption could decrease iron and zinc deficiencies (Christensen et al., 2006; Rumpold and Schlüter, 2013). Aside from their high mineral contents, they could be a source of highly bioavailable iron (Latunde-Dada et al., 2016).

Many complementary porridges consumed in Africa are fermented but still remain high in mineral binders and as such are of low iron and zinc bioavailability (Gabaza et al., 2017b; Towo et al., 2006). The objective of this study is thus to investigate the impact of adding mineral enhancers (ascorbic acid rich baobab fruit pulp powder or meat in the form of mopane worm (*Imbrasia belina*) powder) to fermented cereals (maize, sorghum, pearl millet and finger millet) on the iron and zinc bioaccessibility

measured using dialyzability assay. Data from this study may provide crucial information pertaining to the iron and zinc bioaccessibility of baobab fruit pulp and mopane worm enriched fermented cereal products and possibly pave way for the innovation of new food products thereby increasing the utilization of indigenous food sources.

7.3 Materials and methods

7.3.1 Materials

Cereal grains (maize, red and white sorghum, pearl millet and red and white finger millet) and water used in the preparation of fermented porridges were provided by farmers from Hwedza communal area, Zimbabwe. Baobab fruit pulp powder and mopane worms were purchased from TM Supermarket, Victoria Falls, Zimbabwe. Folin Ciocalteu's reagent, gallic acid, vanillin, catechin, α-amylase from porcine pancreas (Type VI-B, > 10 units/mg solid), pepsin from porcine gastric mucosa (3200-4500 units/mg protein), pancreatin from porcine pancreas (8xUSP), dialysis bags (MW cut off 12-14 kDa), bile from porcine bile extract, phytic acid dodecasodium salt, 2,2'bipyridine and thioglycolic acid were procured from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA) while ICP multi-element standard solution IV was purchased from Merck (Germany).

7.3.2 Methods

7.3.2.1 Preparation of fermented slurries

Fermented cereals were prepared such that they simulated the preparation process done in Hwedza communal area. The fermentation was done using the water that is used for drinking and cooking purposes in this area. The grains were additionally dried overnight at 60^{o}C in an oven followed by milling using a laboratory hammer mill to equipped with sieve of size 0.5 mm. Flour and water were mixed in the ratio 1:3 (w/v) in previously sterilized glass jars and spontaneous fermentation was allowed for 26 hours at 25^{o}C according to the normal practices (Gabaza et al., 2016). During the fermentation, the pH was carefully monitored and at the end of the fermentation, pH ranged between 4.12-5.70. After fermentation, the slurries were transferred to 50 mL sterile falcons and stored at -20^{o}C awaiting transportation to Belgium. The mopane worm was milled to a particle size of 0.5 mm using a laboratory mill and then stored in sterile plastic bags. The fermented cereals, baobab fruit pulp and mopane worm were then freighted under dry ice to the Laboratory of Microbiology and Biotechnology, Ghent University, Belgium during the period September 14-19, 2016. Once in Belgium, enriched fermented cereals were prepared by mixing either the mopane or baobab fruit pulp powder with the fermented cereal in the ratio 1:10 fresh weight basis. This ratio was chosen as the minimum

proportion used when other ingredients are added to porridges based on observations from previous studies (Gabaza et al., 2017b). The mixture was mixed until it was homogenous and then analyzed for mineral contents, mineral binders and subsequently iron and zinc bioaccessibility. Three types of fermented cereals were prepared namely, non-enriched fermented cereals (NEC – partly described in chapter 4 as fermented cereals originating from Hwedza), baobab fruit pulp enriched fermented cereals (BEC) and mopane worm enriched fermented cereals (MEC). All samples were prepared in three independent trials.

7.3.2.2 Analysis

Determination of dry matter content, mineral inhibitor contents (PA, PC and CT) and *in vitro* iron and zinc bioaccessibility was executed according to the methods described in section 3.3.2. Mopane worm and baobab fruit pulp were also analyzed for additional minerals i.e. sodium, magnesium, calcium and potassium. Bioaccessibility was defined as the proportion of minerals able to through a dialysis membrane of 12-14 kDa molecular weight cut-off.

7.3.2.3 Determination of the ascorbic acid content

Ascorbic acid content was only determined in the baobab fruit pulp powder. Ascorbic acid was determined using the dichlorophenolindophenol (DCPIP) method. Baobab fruit pulp powder (2.5 g) was dissolved in 20 mL distilled water and an aliquot of 2 mL from this suspension was titrated against DCPIP until a pink color which lasted for about 5 seconds. Ascorbic acid content was expressed as mg/100 g dm.

7.3.2.4 Statistical Analysis

Data were subjected to two way ANOVA and in all cases there was significant interaction between type of cereal fermented slurry and type of enrichment as such simple effects were considered. In the case of significant differences, comparisons of means was done using Tukey's post-hoc test ($p < 0.05$). Analysis was carried out using IBM SPSS software version 23. Data is shown as means ± standard deviations of three independent samples.

7.4 Results

7.4.1 Characteristics of mopane worm and baobab fruit pulp powder

Table 7.1 shows the results of the characteristics of the mopane worm and baobab fruit pulp in terms of mineral binders (PC, CT and PA), mineral contents (iron, zinc, sodium, magnesium, calcium and potassium), mineral enhancers (vitamin C), and mineral bioaccessibility. Soluble PC and CT were higher in baobab fruit pulp than mopane worm while the amount of bound PC was comparable. In terms of PA content, both mopane worm and baobab fruit pulp did not have detectable PA. The ascorbic acid content of baobab fruit pulp was 64.4 mg/100 g dm. Pertaining to the iron and zinc contents, mopane worm had a higher iron and zinc content than baobab fruit pulp. Both mopane worm and baobab fruit pulp had high contents of sodium, magnesium, calcium and potassium. The %SND iron and zinc of baobab fruit pulp accounted for up to 62% of the total iron and zinc while it accounted for not more than 20% of the total iron and zinc in mopane worm, respectively. Baobab fruit pulp had a higher bioaccessibility (dialyzable minerals, < 14 kDa) of both iron (7.21%) and zinc (12%) compared to mopane worm with iron bioaccessibility of 3.93% and zinc bioaccessibility of 0.61%.

Table 7.1: Characteristics of mopane worm and baobab fruit pulp

Component	Mopane worm	Baobab fruit pulp
Soluble phenolic compounds (mg GAE/100g dm)	126±5	850±177
Bound phenolic compounds (mg GAE/100 g dm)	2075±48	2260±165
Condensed tannins (mg CE/100 g dm)	37.6±7.4	1330±125
Phytic acid (mg/100 g dm)	nd	nd
Vitamin C (mg/100 g dm)	-	64.4±2.0
Sodium (mg/100 g dm)	64.8±6.7	70.3±7.2
Magnesium (mg/100 g dm)	187±1	114±8
Calcium (mg/100 g dm)	221±2	209±14
Potassium (mg/100 g dm)	1093±50	1305±100
Iron (mg/100 g dm)	13.2±0.2	2.45±0.14
Zinc (mg/100 g dm)	9.76±0.28	0.45±0.05
Iron bioaccessibility (%)	3.93±0.64	7.21±2.77
Iron bioaccessibility (mg/100 g dm)	0.52±0.01	0.18±0.01
Zinc bioaccessibility (%)	0.61±0.06	12.0±3.5
Zinc bioaccessibility (mg/100 g dm)	0.06±0.01	0.05±0.01

Limit of detection for phytic acid was 9.09 µg/ml. GAE: gallic acid equivalents, CE: catechin equivalents.

7.4.2 Content of mineral binders in cereals

The higher content of soluble PC in baobab fruit pulp caused BEC to have higher levels of soluble PC (270-992 mg/100 g dm) than the MEC (175-464 mg/100 g dm (**Table 7.2**). As the bound PC of both mopane and baobab fruit pulp was higher than that of the individual NEC, bound PC increased for both BEC and MEC. Moreover, soluble, bound and total PC increased for all the enriched fermented cereals. The contribution of the soluble PC to total PC increased in all enriched cereals from 10.9% to 32.2% for MEC and from 20.6% to 31.9% for BEC.

As a consequence of the high level of CT in baobab fruit pulp and negligible content in mopane worm, BEC had a higher CT which increased from not detected to 957 mg CE/100 g dm while CT in MEC was decreased from 235 to 75.6 mg CE/100 g dm for red finger millet and from 957 to 301 mg CE/100 g dm for red sorghum (**Table 7.3**). Red sorghum fermented slurries had the highest CT in all cases.

Table 7.4 shows the results of the PA content. Since both mopane worm and baobab fruit pulp did not have any PA, all enriched cereals had a reduced PA. PA content ranged from 392-846 mg /100 g dm for the NEC and was reduced by a factor of 31-75% and 22-77% in MEC and BEC, respectively. In all cases, maize based slurries had the highest PA content.

Table 7.2: Soluble and bound PC of fermented cereals

	NEC	MEC	BEC	P value
		Soluble PC		
Maize	$91.5\pm2.1^{a,B}$	$246\pm10^{b,B}$	$285\pm10^{c,A}$	<0.001
Red sorghum	$671\pm26^{b,E}$	$464\pm15^{a,E}$	$992\pm47^{c,C}$	<0.001
White sorghum	$50.5\pm9.4^{a,A}$	$239\pm6^{b,B}$	$297\pm20^{c,A}$	<0.001
Pearl millet	$160\pm10^{a,D}$	$375\pm12^{b,D}$	$527\pm73^{c,B}$	<0.001
Red finger millet	$126\pm4^{a,C}$	$285\pm4^{b,C}$	$481\pm6^{c,B}$	<0.001
White finger millet	$51.1\pm7.5^{a,A}$	$175\pm3^{b,A}$	$270\pm9^{c,A}$	<0.001
P value	<0.001	<0.001	<0.001	
		Bound PC		
Maize	$613\pm22^{a,B}$	$1156\pm207^{b,A,B}$	$1097\pm52^{b,A}$	0.003
Red sorghum	$1417\pm20^{a,D}$	$2239\pm256^{b,C}$	$2117\pm134^{b,C}$	0.002
White sorghum	$349\pm60^{a,A}$	$1016\pm20^{b,A,B}$	$780\pm56^{c,A}$	<0.001
Pearl millet	$659\pm17^{a,B}$	$1084\pm31^{b,A,B}$	$1124\pm59^{b,A}$	<0.001
Red finger millet	$835\pm10^{a,C}$	$1495\pm136^{b,B}$	$1596\pm188^{b,B}$	0.001
White finger millet	$417\pm28^{a,A}$	$878\pm56^{c,A}$	$753\pm41^{b,A}$	<0.001
P value	<0.001	<0.001	<0.001	

NEC: non enriched cereals, MEC: mopane worm enriched cereals, BEC: baobab fruit pulp enriched cereals, PC: phenolic compounds. Values are expressed in mg GAE/100 g dm. Values with different capital superscript letters within columns are significantly different. Values with different small superscript letters across rows are significantly different, $p < 0.05$, n=3. GAE: gallic acid equivalents.

Table 7.3: Level of condensed tannins of fermented cereals

	NEC	MEC	BEC	P value
Maize	ND	$15.3\pm3.2^{a,A}$	$191\pm7^{b,A}$	<0.010
Red sorghum	$957\pm25^{b,B}$	$301\pm15^{a,C}$	$2012\pm127^{c,C}$	<0.001
White sorghum	ND	$21.4\pm24^{a,A}$	$235\pm17^{b,A}$	<0.001
Pearl millet	ND	$7.11\pm2.03^{a,A}$	$245\pm8^{b,A}$	<0.001
Red finger millet	$235\pm10^{b,A}$	$75.6\pm3.7^{a,B}$	$540\pm23^{c,B}$	<0.001
White finger millet	ND	$17.9\pm6.2^{a,A}$	$273\pm7^{b,A}$	<0.001
P value	<0.001	<0.001	<0.001	

NEC: non enriched cereals, MEC: mopane worm enriched cereals, BEC: baobab fruit pulp enriched cereals. Values are expressed in mg CE/100 g dm. Values with different capital superscript letters within columns are significantly different. Values with different small superscript letters across rows are significantly different, $p < 0.05$, n=3. CE: catechin equivalents.

Table 7.4: Phytic acid content of fermented cereals

	NEC	MEC	BEC	P value
Maize	$819\pm52^{c,B,C}$	$564\pm2.3^{b,C}$	$431\pm13^{a,B}$	<0.001
Red sorghum	392 ± 87^{A}	161 ± 86^{A}	$307\pm53^{A,B}$	0.151
White sorghum	$846\pm153^{b,B,C}$	$392\pm39^{a,B}$	$363\pm81^{a,A,B}$	0.002
Pearl millet	$743\pm19^{c,B,C}$	$245\pm9^{b,B}$	$174\pm25^{a,A}$	<0.001
Red finger millet	$541\pm44^{b,A,B}$	$134\pm25^{a,A}$	$138\pm39^{a,A}$	<0.001
White finger millet	$661\pm133^{b,B,C}$	$333\pm26^{a,B}$	$328\pm36^{a,A,B}$	0.004
P value	<0.001	<0.001	<0.012	

NEC: non enriched cereals, MEC: mopane worm enriched cereals, BEC: baobab fruit pulp enriched cereals. Values are expressed in mg/100 g dm. Values with different capital superscript letters within columns are significantly different. Values with different small superscript letters across rows are significantly different, $p < 0.05$, n=3.

7.4.3 Iron and zinc contents

The iron contents ranged from 3.22-13.6 mg/100 g dm in NEC with the highest iron content observed for pearl millet **(Table 7.5)**. Zinc contents of NEC cereals were also higher for pearl millet ranging from 1.25-4.39 mg/100 g dm. On addition of either baobab fruit pulp or mopane worm, generally no effect on iron and zinc content was observed for BEC while an increase was observed in MEC except for pearl millet as a result of the higher iron and zinc content of mopane worm compared to baobab fruit pulp.

Iron content for MEC ranged between 6.80-14.5 mg/100 g dm while zinc content ranged between 3.95-9.14 mg/100 g dm.

Table 7.5: Iron and zinc contents of fermented cereals

	NEC	MEC	BEC	P value
		Iron		
Maize	3.22±0.09^{a,A}	10.2±0.4^{b,B}	3.94±0.33^{a,A}	<0.001
Red sorghum	8.09±0.04^{B}	10.1±2.1^{B}	8.24±0.76^{B}	0.209
White sorghum	4.73±1.04^{A,a}	10.9±0.3^{b,B}	3.66±0.44^{a,A}	<0.001
Pearl millet	13.6±0.8^{C}	14.5±0.9^{C}	13.2±1.1^{C}	0.281
Red finger millet	4.24±0.21^{a,b,A}	6.87±1.34^{b,A}	4.87±0.17^{b,A}	0.033
White finger millet	4.76±1.09^{a,A}	8.14±0.42^{b,A,B}	3.85±0.03^{a,A}	0.003
P value	<0.001	<0.001	<0.001	
		Zinc		
Maize	3.17±0.27^{a,B}	8.81±1.74^{b,B,C}	1.88±0.21^{a,A}	0.002
Red sorghum	1.56±0.69^{a,A}	4.58±1.02^{b,A,B}	1.15±0.02^{a,A}	0.002
White sorghum	1.90±0.44^{a,A}	4.77±0.22^{b,A,B}	1.25±0.15^{a,A}	<0.001
Pearl millet	4.39±0.04^{a,C}	9.14±2.22^{b,C}	4.16±0.17^{a,B}	0.005
Red finger millet	1.70±0.36^{a,A}	3.95±0.92^{b,A}	1.31±0.12^{a,A}	0.003
White finger millet	1.25±0.12^{a,A}	5.18±1.62^{b,A,B}	1.34±0.42^{a,A}	0.004
P value	<0.001	<0.001	0.003	

NEC: non enriched cereals, MEC: mopane worm enriched cereals, BEC: baobab fruit pulp enriched cereals. Values are expressed in mg/100 g dm. Values with different capital superscript letters within columns are significantly different. Values with different small superscript letters across rows are significantly different, $p < 0.05$, n=3.

7.4.4 Iron and zinc bioaccessibility

The results of the iron and zinc bioaccessibility are shown in **Table 7.6 and 7.7,** respectively**.** The %SND for iron ranged from 25.8-65.0% for NEC and was lowest for red sorghum and highest for maize. A variable response was observed after the addition of either baobab fruit pulp or mopane worm. In relation to NEC, addition of mopane worm resulted in a decrease of %SND iron for all cereals except for white sorghum where it remained the same. The effect of baobab fruit pulp was less consistent and depended on the type of cereal i.e. an increased %SND was observed for BEC of pearl millet, white

sorghum and red finger millet while a marginal decrease was measured for maize, red sorghum and white finger millet.

Bioaccessibility of iron (estimated by the proportion of dialyzable iron, < 14 kDa), ranged from 7.08-22.7% for NEC with highest bioaccessibility observed for white sorghum and lowest for pearl millet. Bioaccessibility of enriched cereals ranged from 5.31-33.1% and 8.42-44.9% in MEC and BEC, respectively. Maize recorded the highest iron bioaccessibility in both MEC and NEC. Iron bioaccessibility was increased for BEC of maize, red sorghum and white finger millet while no change was observed for other cereals although the %SND of these cereals had been increased. Generally, for BEC, cereals with increased %SND iron, had reduced iron bioaccessibility and vice versa. For the MEC, iron bioaccessibility was increased for maize, pearl millet and red finger millet while it was reduced for white sorghum and no effect was observed for white finger millet and red sorghum. Perhaps the overall effect of adding either baobab fruit pulp or mopane worm can be elucidated from the total soluble iron (%SND+D) which was generally reduced for MEC (33.9-79.1% to 22.0-68.9%) while it was increased for BEC from 33.9-79.1% to 46.1 to 79.7% except for maize where no changes were observed.

Pertaining to zinc, %SND for the NEC ranged from 22.4 to 30.9%. There was a decrease in %SND for MEC for all cereals. A variable response was observed for BEC, i.e. an increase in %SND for white sorghum, pearl millet and red finger millet while a decrease was observed for maize and red sorghum and no effect for white finger millet. Zinc bioaccessibility ranged from 0.74 to14.4% for NEC, 1.00 to 18.2% for MEC and 9.25 to 25.9% for BEC. An increase in zinc bioaccessibility was observed for BEC of maize, red sorghum and white finger millet. In the case of MEC, a decrease or no change in bioaccessibility was observed. A similar trend was also observed for zinc whereby the total soluble zinc (%SND+D) was reduced in all cases where mopane worm was involved (31.6-48.1% to 5.21-24.5%) whilst it was increased in all BEC (31.6-48.1% to 37.2-58.6%) except for maize where it remained the same.

Table 7.6: Soluble non dialyzable and bioaccessible (dialyzable) iron (%) of fermented cereals

	NEC	MEC	BEC	p values
		SND iron		
Maize	65.0±13^{B}	35.8±4.3^{B}	31.2±14.1^{A}	0.054
Red sorghum	25.8±6.9^{b,A}	16.7±0.2^{a,A}	19.4±1.7^{a,A}	0.007
White sorghum	36.5±11.1^{a,A,B}	34.5±6.3^{a,B}	62.3±7.6^{b,B}	0.012
Pearl millet	51.5±6.1^{b,A,B}	18.5±2.8^{a,A}	59.7±7.5^{b,B}	<0.001
Red finger millet	53.8±0.3^{B,a,B}	37.9±2.9^{a,B}	69.5±5.5^{c,B}	<0.001
White finger millet	47.6±6.8^{A,B}	32.8±8.7^{B}	39.9±7.1^{A,B}	0.092
P value	0.015	<0.001	0.001	
		Bioaccessible (dialyzable) iron		
Maize	14.1±1.8^{a,A,B}	33.1±0.1^{b,C}	44.9±1.7^{c,C}	<0.001
	(0.45)	(3.38)	(1.76)	
Red sorghum	8.12±1.72^{a,A}	5.31±0.76^{a,A}	26.7±5.2^{b,B}	0.001
	(0.66)	(0.54)	(2.20)	
White sorghum	22.7±9.1^{B}	12.2±0.5^{A,B}	17.1±2.6^{A,B}	0.245
	(1.07)	(1.33)	(0.63)	
Pearl millet	7.08±0.68^{a,A}	20.8±4.4^{b,B,C}	8.42±2.03^{a,A}	0.002
	(0.96)	(3.02)	(1.11)	
Red finger millet	9.46±0.22^{a,A}	29.2±7.6b^{C}	10.2±4.6^{a,A}	0.005
	(0.40)	(2.00)	(0.50)	
White finger millet	12.6±2.7^{A,B}	12.0±5.6^{A,B}	25.2±7.4^{A}	0.050
	(0.60)	(0.98)	(0.97)	
P value	0.001	<0.001	<0.001	

NEC: non enriched cereals, MEC: mopane worm enriched cereals, BEC: baobab fruit pulp enriched cereals, SND: soluble non dialyzable. Values with different capital superscript letters within columns are significantly different. Values with different small superscript letters across rows are significantly different. Values in parenthesis are mean values of dialyzable (bioaccessible) contents of iron (mg/100 g dm), $p < 0.05$, n=3.

Table 7.7: Soluble non dialyzable and bioaccessible (dialyzable) zinc (%) of fermented cereals

	NEC	MEC	BEC	P value
		SND zinc		
Maize	29.7±4.9^{b,A,B,C}	13.1±1.5^{a,B,C}	15.2±4.6^{a,A}	0.004
Red sorghum	30.9±2.5^{c,B,C}	3.2±0.6^{a,A}	19.9±1.6^{b,A,B}	<0.001
White sorghum	22.8±5.0^{a,b,A,B}	13.0±2.7^{a,b,B,C}	30.5±6.0^{b,B,C}	0.012
Pearl millet	37.3±1.9^{b,C}	20.1±0.5^{a,D}	49.4±3.0^{c,D}	<0.001
Red finger millet	22.4±1.0^{b,A}	10.0±0.6^{a,B}	33.1±3.7^{c,C}	<0.001
White finger millet	22.7±0.3^{b,A,B}	14.5±0.7^{a,C}	21.8±3.2^{b,A,B}	0.015
P value	<0.001	<0.001	<0.001	
		Bioaccessible (dialyzable) zinc		
Maize	1.67±0.36^{a,A}	2.42±0.08^{a,A}	15.3±1.4^{b,A}	<0.001
	(0.05)	(0.21)	(0.29)	
Red sorghum	0.74±0.45^{a,A}	2.01±0.42^{a,A}	17.3±5.6^{b,A,B}	0.006
	(0.01)	(0.09)	(0.20)	
White sorghum	11.8±5.1^{b,B}	1.00±0.02^{a,A}	13.1±4.0^{b,A}	0.040
	(0.22)	(0.05)	(0.16)	
Pearl millet	10.8±1.4^{b,B}	4.43±0.23^{a,A}	9.25±1.97^{b,A}	0.015
	(0.47)	(0.40)	(0.38)	
Red finger millet	14.4±2.4^{B}	18.2±7.6^{B}	9.80±2.83^{A}	0.170
	(0.24)	(0.72)	(0.13)	
White finger millet	12.8±1.8^{b,B}	3.13±1.81^{a,A}	25.9±0.4^{c,B}	<0.001
	(0.16)	(0.16)	(0.35)	
P value	<0.001	0.002	0.003	

NEC: non enriched cereals, MEC: mopane worm enriched cereals, BEC: baobab fruit pulp enriched cereals, SND: soluble non dialyzable. Values with different capital superscript letters within columns are significantly different. Values with different small superscript letters across rows are significantly different. Values in parenthesis are mean values of dialyzable contents of zinc (mg/100 g dm), $p < 0.05$, n=3.

7.4.5 Potential contribution (%) of porridges towards the absolute requirements and recommended dietary allowance of iron and zinc

The contribution that the porridges can potentially make towards the absolute requirements and RDA (recommended dietary allowance) of iron and zinc was calculated for children aged between 1-3 years using the assumption that at the maximum, a child consumes a total of 100 g porridge, 20% dry matter three times a day or 150 g porridge, 20% dry matter, twice a day (Gabaza et al., 2017b). Absolute requirements were calculated using bioaccessibility results (assuming that bioaccessibility measured in our study is a good predictor of bioavailability) while RDA was calculated using total mineral contents **(Figure 7.1).** NEC contributed lower iron to the absolute requirement (41-111%) compared to 51-227% in BEC and 55-349% in MEC. With respect to the RDA of iron, NEC and BEC contributed almost the same level of iron ranging from 33-140% while much higher contribution was observed from MEC (71-150%). Pertaining to zinc, lower contribution to the absolute requirement was observed for all the cereals i.e. NEC (0.6-23%), BEC (6.4-17%) and MEC (2.4-35%). The contribution to the RDA of zinc was highest for MEC (11-66%) compared to 8.3-31 % for NEC and BEC.

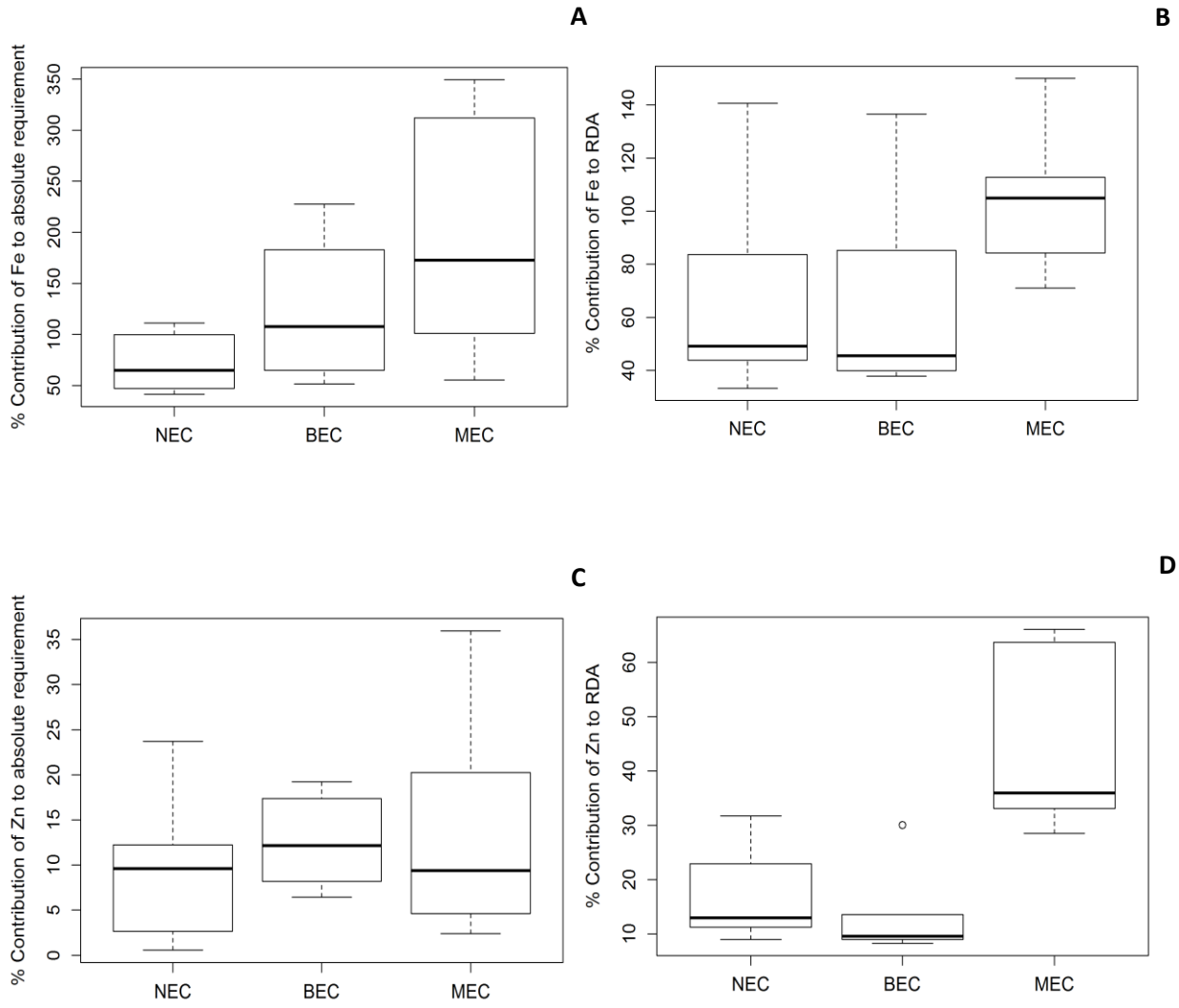


Figure 7.1: Contribution (%) of fermented cereals towards the iron absolute requirements (A), iron RDA (B), zinc absolute requirements (C) and zinc RDA (D).

NEC: non enriched cereals, MEC: mopane worm enriched cereals, BEC: baobab fruit pulp enriched cereals, AR: absolute requirement, RDA: recommended dietary allowance. Absolute requirement is defined as the nutrient requirement for growth and basal losses. Maximum consumption of 100 g portion of porridge, 20% dry matter, 3 times a day was considered. Absolute requirement for iron is 0.58 mg/day for children between the ages 1-3 years while for zinc it is 1.2 mg/day for the same age group. Recommended dietary allowance for zinc is 8.3 mg/day (assuming 15% bioaccessibility) for iron it is 5.8 mg/day (assuming 10% bioaccessibility) (World Health Organization, 2004).

7.5 Discussion

To our knowledge, this is one of the first studies to investigate the effect of enriching commonly consumed cereals in Africa with either baobab fruit pulp or mopane worm on the bioaccessibility of iron and zinc. Enrichment of cereals with indigenous products shows great potential in terms of improving macro and micro nutrients of the resultant food products. This has been shown to be successful when baobab fruit pulp was composited with either sorghum or millet, resulting in an increase in the bioaccessibility of pro-vitamin A from 18.3-31.7% (Debelo et al., 2016). Maize composited with green leafy vegetables such as amaranth, pumpkin and spider plant showed an increased bioaccessible iron and zinc content from 0.29 to 1.12-2.13 mg/100 g and 0.16 to 0.63-1.19 mg/100 g dm, respectively (Kruger et al., 2015). In another study by Lung'aho and Glahn (2009), compositing cereals normally consumed in Kenya i.e. maize, sorghum and finger millet, during the preparation of complementary porridges with local ingredients such as cassava, increased the iron bioavailability.

The bioaccessibility of iron and zinc of the enriched products will depend on the overall interaction of mineral binders (PA, PC and CT) and mineral enhancers such as ascorbic acid with their matrix. Pertaining to the total PC, they were increased in all the enriched cereals as the baobab fruit pulp and mopane worm contained high amounts of both soluble and bound PC but particularly higher in baobab fruit pulp (Table 7.2). Baobab fruit pulp is well known for its high PC content which ranged from 1870-4057 mg GAE/100 g (Coe et al., 2013; Lamien-Meda et al., 2008; Tembo et al., 2017) and this agrees with our finding of total PC (3110 mg GAE/100 g) (Table 7.1). Although edible insects can be classified as animal meat, phytochemicals have been identified in several edible insects. It is suggested that quite a number of edible insects have the ability to sequester compounds from host plants and store them as defense mechanisms or use them as precursor molecules for the biosynthesis of other compounds (Musundire et al., 2014b). Total PC amounting to 3600 mg GAE/100 g have been reported from Zimbabwean stinkbugs while 777 mg GAE/100 g were observed from ground crickets (Musundire et al., 2014a; Musundire et al., 2014b). In addition to PC, CT increased in all BEC while a reduction in CT was observed for MEC (Table 7.3). A recent study by Tembo et al. (2017) confirmed the presence of procyanidin B_2 in the baobab fruit pulp pulps from Malawi amounting to 533 mg/100 g while CT ranging from 17-310 mg CE/100 g has been found in some edible insects (Musundire et al., 2014a; Musundire et al., 2014b). PA was reduced in all BEC and MEC as there was no detected PA in both baobab fruit pulp and mopane worm (Table 7.4). This finding agrees with reports from other authors as well who found negligible PA in insects and baobab fruit pulp (Chadare et al., 2008; Musundire et al., 2014b). Although it was expected for the enriched cereals to have decreased PA due to the absence of PA in both the mopane worm and baobab fruit pulp, the magnitude of change did not conform with the 1:10

proportion that was used for enrichment. Phytic acid reductions of up to 77% were observed in contrast to expected reductions of not more than 40%. This could be a result of interactions between the cereal and the mopane worm or baobab fruit pulp leading to reduced recovery of PA. Mopane worm contains proteins that could have interfered with the PA analysis due to the interaction between PA and protein leading to reduced extraction of PA (Reichwald and Hatzack, 2008). Additionally, both mopane worm and baobab fruit pulp contain high calcium, magnesium, potassium and sodium (Table 7.1) and up to 40% fiber (Kaboré et al., 2011; Madibela et al., 2009) and PA-mineral-fiber interactions could have reduced recovery of PA (Lopez et al., 2002).

The enrichment of cereals with indigenous food sources can only improve iron and zinc nutrition if they increase the total mineral contents and/or they add mineral enhancers such as ascorbic acid. In terms of mineral contents, only MEC had increased iron and zinc (Table 7.5) as it has been reported by several authors that insects are excellent sources of iron and zinc (Christensen et al., 2006; Ghosh et al., 2017; Rumpold and Schlüter, 2013). However, higher than expected iron and zinc contents for some MEC in particular maize, were observed (up to 80% higher than expected) thus these results should be interpreted with caution. Mopane worm, unlike the baobab fruit pulp, could not sufficiently dissolve in the fermented slurries in order to form a homogenous mixture which would enable easy sampling for analysis in the same 1:10 ratio that was used for enrichment. Nonetheless, based on the higher iron and zinc content of the mopane worm than most of the cereals, an increase in iron and zinc content of MEC was to be expected. Iron and zinc content ranging from 1.1-10.4 mg/100 g and 0.5-3.2 mg/100 g dm respectively, were reported for baobab fruit pulp showing that baobab fruit pulp can also be a good source of iron but not of zinc. Ascorbic acid, a potent enhancer of iron bioavailability, was added in all BEC as baobab fruit pulp is a good source of ascorbic acid. However, the baobab fruit pulp used in this study also had lower level of ascorbic acid (64.4 mg/100 g dm) compared to 150-500 mg/100 g reported by several authors (Chadare et al., 2008; Parkouda et al., 2012). As the baobab fruit pulp used was not freshly harvested, it can be inferred that losses of ascorbic acid occurred during storage as ascorbic acid is not stable and sensitive to photo-oxidation (Tembo et al., 2017).

An assessment of the enriched cereals indicates that there was an increase in PC for all cereals, increase in CT of BEC, reduction of PA in all cereals and increase of iron and zinc in MEC (Table 7.2-7.5). Additionally, BEC contained ascorbic acid from the baobab fruit pulp. The effect of this enrichment process was an increase in the total soluble iron of BEC. Increase in total soluble iron of BEC could likely be the result of ascorbic acid which is known to overcome the effects of PA and PC. It has been established that ascorbic acid increases the absorption of iron (Cercamondi et al., 2014b). A prerequisite for iron to be absorbed is its solubility at the physiological pH of the intestine. At acid pH (between pH 2-6), Fe^{3+} is reduced during gastric digestion with the assistance of ascorbic acid forming

Fe^{2+} which is highly bioavailable at the physiological pH of the intestine (Cercamondi et al., 2014b; Engle-Stone et al., 2005; Hsieh and Hsieh, 1997). The action of ascorbic acid during gastric digestion may thus assist in the dissociation of iron from mineral binders. The reducing effect of ascorbic acid is important as Fe^{3+} rapidly becomes insoluble at pH above 3 (Cercamondi et al., 2014b). Ascorbic acid also forms soluble and weak complexes with iron at the intestinal pH and these complexes are subsequently bioavailable in the absence of compounds that may bind iron (Engle-Stone et al., 2005). Furthermore, the presence of Fe^{2+} at intestinal pH increases iron absorption as Fe^{2+} forms much weaker complexes with PC than Fe^{3+} such that it is easily absorbed (Cercamondi et al., 2014b). Although the total soluble iron was increased in almost all the cereals, the bioaccessibility (dialyzable iron of < 14 kDa) was not increased in all cereals possibly due to matrix differences and the presence of different structures of PC which have different iron binding power in the different cereal grains (Table 7.6). Nonetheless, an increase in the total soluble iron is a positive effect on bioaccessibility.

According to the composition of BEC and the content of ascorbic acid in the baobab fruit pulp used in this study (64.4 mg/100 g dm), a 100 g serving of enriched porridge will contain about 3.22 mg/100 g dm ascorbic acid. This amount of ascorbic acid is too low compared to the amounts reported by other authors to be crucial for ascorbic acid to reverse the effects of PA and PC for example, 17 mg/100 g ascorbic acid were reported by Cercamondi et al. (2014b) to increase bioavailability of iron in red sorghum from 2.70-13.6%, 30 mg ascorbic acid were needed to overcome effects of 10-58 mg PA in wheat bread while more than 50 mg ascorbic acid were needed to overcome the effects of 100 mg tannic acid (Siegenberg et al., 1991). Teucher et al. (2004) further suggested that a molar ratio of 2:1 ascorbic acid: Fe (20 mg ascorbic acid, 3 mg iron) is needed for foods with low-medium levels of inhibitors while a molar excess of 4:1 is required for foods with high level of inhibitors. In general, ascorbic acid enhances iron bioavailability in a dose dependent manner thus the increase in iron bioaccessibility in BEC may not be an effect of ascorbic acid alone but possibly other components and their interactions in baobab fruit pulp may have a synergistic enhancing effect on iron bioaccessibility. This is also demonstrated by the fact that zinc bioaccessibility of BEC was generally improved as well, even though it has been suggested that ascorbic acid does not have an effect on zinc bioavailability (Solomons et al., 1979).

Aside from ascorbic acid, the positive effect of baobab fruit pulp on iron and zinc bioaccessibility could have emanated from other organic acids such as citric, tartaric, malic and succinic acid which have been found to give the baobab fruit pulp an acidic nature with pH around 3.3 (Chadare et al., 2008). A positive effect but of varying degrees on iron bioavailability was reported after addition of nine organic acids namely tartaric, malic > succinic, fumaric > citric, lactic > acetic, propionic acid (Salovaara et al., 2002). The addition of citric acid and amchar (Indian spice containing combination of citric acid and

malic acid) to cereals and legumes such as rice and chickpea increased the iron and zinc bioaccessibility by up to 60% (Hemalatha et al., 2005). The enhancing effect of organic acids is likely as a result of their functioning as chelates such that they form soluble complexes with the minerals (Salovaara et al., 2002). The bioaccessibility of these soluble complexes is then influenced by factors such as the strength of the complex and its interaction with its matrix as seen from the differences in the iron and zinc bioaccessibility of the different cereals (Table 7.6 and 7.7). As a result of the acidic nature of baobab fruit pulp, its addition to the fermented cereals could have further increased the buffering capacity of BEC thereby providing an optimal environment for the solubilization of iron. The addition of small amounts of lactic acid to maize products increased the solubility and subsequent bioaccessibility of iron (Proulx and Reddy, 2007). The enhancing effect of baobab fruit pulp could thus be attributed to the synergistic effect of ascorbic acid, organic acids and buffering capacity which is additional to the acids produced during fermentation.

A contrasting effect on iron and zinc bioaccessibility was observed in the case of MEC. The enhancing effect of meat known as the “meat factor” or meat effect” (Baech et al., 2003; Engle-Stone et al., 2005) was not observed in this study suggesting that this effect may not be present in mopane worms (Table 7.6 and 7.7). The mechanism behind the meat factor still remains elusive but it has been proposed that small sulfated glycosaminoglycan carbohydrates (Huh et al., 2004) and also sulfhydryl amino acids like cysteine and peptides are involved (Baech et al., 2003). The “meat factor” is thus not about haem iron but about specific proteins and carbohydrates associated with meat. Haem iron cannot be measured using the dialyzability assay as haemoglobin and myoglobin cannot pass through a dialysis membrane of 12-14 kDa. Nonetheless, if haem iron was present in the mopane worm, it should be measured in the soluble non dialyzable fraction. In addition, if the meat factor was present in mopane worm, it should at the minimum, increase the soluble fraction of iron in MEC but this was not the case. A study by Latunde-Dada et al. (2016) revealed that among four insects; grasshopper, cricket, mealworm and buffalo worm, only buffalo worm had higher and comparable iron bioavailability with that of sirloin beef. In that light, the iron bioaccessibility of insects may be quite diverse and more insight is required pertaining to the chemical composition of iron in edible insects. Another plausible reason for the general negative effect of MEC on iron and zinc bioaccessibility is the presence of chitin, a non-digestible modified polysaccharide present in many edible insects. Mopane worm contains about 27% (dm basis) chitin (Kwiri et al., 2014) and it is probable that the chitin prevented the digestive enzymes from accessing substrates such that there was a reduced digestibility with consequence on the release of minerals from macromolecules. It has been shown that removal of chitin in insects could increase digestibility of proteins therefore compositing mopane worm with cereals may have potential to improve iron and zinc bioaccessibility if chitin is removed (Verkerk et al., 2007).

The overall interaction of inhibitors and enhancers caused a general negative effect in iron and zinc bioaccessibility of MEC and a general positive effect in BEC. However, the bioaccessible iron contents of MEC still remained somewhat higher or comparable to that of BEC. When estimating the contribution that the bioaccessible minerals can make towards the absolute requirements, it is more reliable to consider the existence of a positive effect rather than the magnitude of that effect (Fairweather-Tait et al., 2005). Contribution of iron to absolute requirements was in the following order: NEC < BEC < MEC while for zinc it was NEC, MEC < BEC (Figure 7.1) implying that baobab fruit pulp could improve zinc nutrition while both baobab fruit pulp and mopane worm could improve iron nutrition for children. The addition of both baobab fruit pulp and mopane worm can contribute positively to iron nutrition due to the increased iron bioaccessibility in BEC and increased iron contents in MEC. It should be noted that the amount of bioaccessible iron and zinc content for some of the MEC in particular maize (Table 7.6 and 7.7, values shown in parenthesis), should not be considered as absolute values as these values were higher than expected due to the inhomogeneity of the sample. However, the fact remains that enriching fermented cereals with mopane worm could increase iron and zinc intake but the magnitude of this increase needs further enquiry. In terms of zinc nutrition, more efforts are required as based on the zinc contents observed in this study, populations subsisting on cereals may be more at risk of zinc than iron deficiency. In this study, a positive effect on zinc bioaccessibility was observed in BEC but this effect was not meaningful as the total zinc contents of the cereals were low. In contrast, MEC had higher zinc contents but very low bioaccessibility such that the amount of bioaccessible zinc remained marginally low. Food based strategies should thus focus on increasing both the zinc contents in food products and also the bioaccessibility which in this case may be done by compositing the cereals with both baobab fruit pulp (for increased zinc bioaccessibility) and mopane (for increased zinc contents). Further studies on this aspect are thus warranted.

7.6 Conclusion

Food-to-food fortification has great potential in improving the iron and zinc nutrition of people living in the poor communities of developing countries. Generally, addition of baobab fruit pulp had a positive effect on the total soluble iron and zinc while mopane worm had a variable effect. There was a variable response to iron and zinc bioaccessibility (defined as the proportion of iron and zinc able to pass through a dialysis membrane of 12-14 kDa molecular weight cut-off) which depended on the type of cereal. Mopane worm increased the iron and zinc contents of the MEC such that the contribution that the MEC can make towards the absolute requirement and RDA of iron and zinc was higher or

comparable to that of the higher bioaccessible BEC. The enrichment of cereals with certain indigenous food sources is thus beneficial and has great potential in alleviating mineral deficiencies.

Chapter 8:

General discussion, future perspectives and conclusions

Chapter 8 : General discussion, future perspectives and conclusions

8.1 Usefulness of dialyzability to predict iron and zinc bioavailability of cereal porridges

Dialyzability assays are widely used to predict mineral bioavailability of many foods and they have found wide usage among many researchers since they are relatively inexpensive, rapid and easy to perform. For dialyzability to predict accurately the bioavailability, it must predict both the correct direction and magnitude of response to food components that modulate iron and zinc bioavailability in human subjects (Aragón et al., 2012). Dialyzability usually, but not always, predicts the correct direction of response (Fairweather-Tait et al., 2007). A major limitation of dialyzability is pertaining to its potential to give misleading information based on the working concept that small dialyzable compounds will be bioavailable. This is because some molecules such as some small soluble PC bound to iron or zinc and small organic acids may be dialyzable but actually not bioavailable while some large molecules such as ferritin may not be dialyzable but be of high bioavailability (Fairweather-Tait et al., 2007; Fairweather-Tait et al., 2005; Luten et al., 1996). In the following two sections, a comparison is made between the obtained iron and zinc dialyzability (referred to as bioaccessibility in this PhD) results of this PhD with bioavailability results of available human studies where cereals (maize, sorghum and millets) were used as test meals.

8.1.1 Iron bioaccessibility

Bioavailability of iron is widely known to be negatively influenced by PA and PC while it can be enhanced by ascorbic acid, meat and some small organic acids. **Table 8.1** shows the iron bioaccessibility results from our study categorized according to the PA content (< 1 and > 1), PC and CT content and also presence of ascorbic acid. According to Hurrell and Egli (2010), PA/Fe < 1 is recommended to have improved iron bioavailability while PA/Fe < 6 is recommended in the presence of ascorbic acid. The amount of ascorbic acid required was not given but according to Teucher et al. (2004), molar ratio of 2:1 ascorbic acid: Fe (20 mg ascorbic acid, 3 mg iron) is needed for foods with low-medium levels of inhibitors while a molar excess of 4:1 is required for foods with high level of inhibitors. Only PA/Fe < 6 and presence of ascorbic acid will be considered irrespective of the amount of ascorbic acid present since the ascorbic acid which was added in the form of baobab fruit pulp powder (Chapter 7) could not meet the requirements described by Teucher et al. (2004). Iron bioaccessibility of cereals (including fermented cereals enriched with baobab fruit pulp or mopane worm and enzyme treated cereals, Chapter 3, 4, 6 and 7) was 1.23-44.9% with median of 12.1%. The lowest iron bioaccessibility of 1.23% was observed for native red sorghum flour whilst the highest bioaccessibility of 44.9% was observed for fermented maize with ascorbic acid added in the form of

baobab fruit pulp. Analogous to our results is the bioavailability of iron from human studies where cereals were used as test meals **(Table 8.2).** Iron bioavailability ranged from 0.64-13.6% (median 2.88%) with lowest bioavailability reported for red sorghum and highest bioavailability reported for red sorghum with ascorbic acid. The dialyzability assay used in our study was able to predict the correct direction of response as observed in the low bioaccessibility of iron from red sorghum because of the inhibitory effect of CT and in the high bioaccessibility when ascorbic acid was present.

Table 8.1: Overview of the iron bioaccessibility of cereals studied in this PhD (Chapter 3, 4, 6 and 7)

PA/Fe	PC	% bioaccessibility[1]	Amount bioaccessible (mg/100 g dm)
< 1	Low	3.28-14.0 (8.89)	1.28-3.98
< 1	High	8.86-28.8 (10.7)	0.06-3.45
> 1	Low	2.77-36.7 (14.01)	0.25-2.69
> 1	high	1.23-32.6 (8.86)	0.27-3.18
< 6 + ascorbic acid	-	8.42-26.7 (10.2)	0.50-2.20
> 6 + ascorbic acid	-	17.1-44.9 (25.2)	0.63-1.76

[1] shows range of values and median in parenthesis. PA: phytic acid, PC: phenolic compounds. High PC is classified as having total PC > 1000 mg GAE/100 g and CT. GAE: gallic acid equivalents. Low PC cereals are maize, pearl millet, white sorghum and white finger millet while high PC cereals are red sorghum and red finger millet. Cereals are either fermented or dephytinized enzymatically and may include some additions of mopane worm. Ascorbic acid was added in the form of baobab fruit pulp powder.

Table 8.2: Bioavailability of iron from available human studies where cereals were used as test meals

Product description	PA/Fe	PC	% Bioavailability	Reference
Maize porridge	> 1	low	1.44-2.25	Hurrell (2004)
Maize porridge (dephytinized)	< 1	low	7.33-10.85	Hurrell (2004)
Sorghum porridge	> 1	high	0.64-1.37	Hurrell (2004)
Sorghum porridge (dephytinized)	< 1	high	0.85-1.85	Hurrell (2004)
Sorghum porridge	> 1	low	1.01-2.27	Hurrell (2004)
Sorghum porridge (dephytinized)	< 1	low	1.68-4.63	Hurrell (2004)
Maize tortillas	> 1	low	1.93	Mendoza et al. (1998)
Maize tortillas (genetically reduced PA)	< 1	low	2.88	Mendoza et al. (1998)
Sorghum porridge	> 1	low	8.5-10.7	Cercamondi et al. (2014b)
Sorghum porridge	> 1	high	2.7-4.6	Cercamondi et al. (2014b)
Sorghum porridge + ascorbic acid	> 1	high	13.6	Cercamondi et al. (2014b)
Pearl millet	> 1	low	5.7-10.0	Cercamondi et al. (2013)
Pearl millet (biofortified)	> 1	low	5.6-10.1	Cercamondi et al. (2013)

Only human studies where maize, sorghum and millets were used as test meals were considered. Other cereals such as rice and wheat were not considered. All the human studies were based on extrinsic radioisotope studies done on adult men and women aged between 18-40 years. Only one study by Cercamondi et al., 2013 was on African adults while other studies were based on European and American adults with a Western type background diet (predominantly low PA diet).

The human studies by Hurrell (2004), **Table 8.2**, show different folds increase in the iron bioavailability after dephytinization of low PC maize and white sorghum. Conversely, non-dephytinized pearl millet had a bioavailability of 5.6-10.1% (Cercamondi et al., 2013) which is in the same range with dephytinized maize. In addition, there exists clear differences between the iron bioavailability of

dephytinized or not dephytinized low PC sorghum and high PC sorghum (Cercamondi et al., 2014b; Hurrell, 2004). These observations purport a matrix dependence on the effect of PA and PC on iron bioavailability which is in tandem with our results whereby the bioaccessibility of iron varied among the cereal matrix used. In this PhD (chapter 3,4,6 and 7), higher iron bioaccessibility was generally observed for maize (median 14.1%), white finger millet (median 13.6%) and white sorghum (median 12.6%) while marginally lower bioaccessibility was observed for red sorghum (median 10%), red finger millet (median 10%) and pearl millet (median 8.79%). The effect of PA on iron bioavailability was not apparent among different human studies suggesting multifaceted factors influencing iron bioavailability besides PA in cereals as also observed in this PhD.

There are marked differences between our bioaccessibility study and human studies pertaining to the magnitude of response. The median iron bioaccessibility from our study (chapter 3,4,6 and 7) was 12.1% which is higher than the median of 2.88% from the human studies. According to Hurrell and Egli (2010), conservative values for non-haem bioavailability are between 5-12% but absolute non-haem absorption can be as low as 1%. In all the human studies mentioned in **Table 8.2**, the iron bioavailability never exceeded 15% even in the presence of ascorbic acid indicating that our bioaccessibility results were higher than *in vivo* results. There are several reasons that could explain the differences in the magnitude of response between *in vitro* and *in vivo* results. One reason is based on the complexity of the redox reactions of iron during digestion which are quite different *in vitro* vs. *in vivo*. The solubility of iron at the physiological pH is heavily reliant on the pH and redox potential and this is quite challenging to simulate correctly *in vitro* because during *in vitro* intestinal digestion, the solid food remains in contact with the soluble components and this is not what transpires *in vivo*. The kinetics of release of iron and the pH-redox changes during *in vitro* digestion need to be fully understood in order for *in vitro* methods to give a good estimation of bioavailability. Another plausible reason is the effect of the dialysis process whereby some iron compounds may be dialyzable but in fact not be bioavailable such as some small phenolic compounds which can bind iron like gallic acid. Hithamani and Srinivasan (2014) showed that gallic acid from finger millet was highly bioaccessible. In addition, there is possibility of some small colloidal particles of insoluble iron being able to pass through the dialysis membrane thereby giving higher dialyzability values than experienced *in vivo*. Many studies have also shown that dialyzability usually results in higher bioavailability values than *in vivo* methods although the direction of response was almost always accurate (Aragón et al., 2012; Walter et al., 2003). It has to be mentioned also that the analysis of iron presents many challenges as it is the most abundant element in the earth, and as such sources of contamination during analysis are numerous. A study by Luten et al. (1996) revealed that the reproducibility of the dialyzability method for *in vitro* iron determination from nine laboratories was only 20-30% which could be a result of the challenges

encountered in iron analysis. There are also some factors that make it difficult to compare *in vitro* vs. *in vivo* methods. It has been suggested that iron bioavailability may depend on the background diet and the iron status of the subjects. Habitual consumption of high phytate diets may reduce the inhibitory effects of phytate on iron absorption (Armah et al., 2015) while an inverse correlation exists between iron status and iron absorption (Hurrell and Egli, 2010).

The available human studies shown in **Table 8.2** do not demonstrate the effect of fermentation. Future studies should thus investigate the bioavailability of fermented cereals as there are many *in vitro* studies which suggest improved iron bioavailability but these studies need to be proven *in vivo*. Furthermore, the effect of contaminant iron is important to consider as in our study, we have shown the presence of highly bioaccessible iron from cereals originating from Chiweshe and lowly bioaccessible iron from cereals originating from Chiredzi (Chapter 4). Effect of consuming iron contaminated cereals *in vivo* also needs further investigation. Consumption of iron contaminated cereals in areas where threshing of cereal grains is practiced has been observed in Ethiopia, Malawi and many other African countries (Harvey et al., 2000). To our knowledge, this study is the first to show iron contamination of cereals from Zimbabwe. A recent study by Gibson et al. (2015) showed the importance of contaminant iron to the nutritional status of Malawian women who habitually consume iron contaminated millet. Henceforth, the effect of fermentation and contaminant iron must be tested *in vivo* and compared against dialyzability studies to determine whether dialyzability can estimate these attributes correctly.

A thorough understanding of all these factors that impact on iron bioavailability may assist in drawing up of correct estimates for iron bioavailability. An estimate of 5% iron bioavailability is normally used for diets based on cereals with no animal protein and ascorbic acid while 10% bioavailability is considered for cereal based diets which include ascorbic acid from fruits and vegetables (Zimmermann and Hurrell, 2007). Since fermentation is known to reduce mineral binders and considered to improve the bioavailability of cereals (Gibson et al., 1997), it can be inferred that the bioavailability of fermented cereals should go beyond 5% and possibly beyond 10% as well. Dephytinization of cereals is known to improve iron bioavailability but for some cereal grains, iron bioavailability still remained below 5% even after complete dephytinization (Hurrell, 2004; Mendoza et al., 1998). Many studies use an estimation of 10% bioavailability to estimate iron requirements in developing countries but it is clear that this estimation may underestimate iron requirements. There is an urgent need for more rigorous standardized studies to allow for better understanding of iron bioavailability under different scenarios.

8.1.2 Zinc bioaccessibility

In **Table 8.3** an overview of the zinc bioaccessibility of cereals from our study (Chapter 3,4,6 and 7) is given, distinguishing three categories of PA/Zn and two categories of PC (high or low). PA is considered as the major inhibitor of zinc absorption as such many human studies investigate this parameter. It has to be mentioned however that most studies on zinc bioavailability in humans use products fortified with zinc such that these studies may not closely mimick our own study but could be albeit useful for comparisons sake. **Table 8.4** shows the zinc bioavailability of available human studies where cereals (maize, sorghum and millets) were used as the test meals.

Table 8.3: Overview of the zinc bioaccessibility of cereals studied in this PhD (Chapter 3, 4, 6 and 7)

PA/Zn	PC	% bioaccessibility[1]	Amount bioaccessible (mg/100 g dm)
> 15	low	0.79-25.9 (11.6)	0.01-0.47
> 15	high	0.45-17.3 (9.13)	0.01-0.22
5-15	low	1.21-11.9 (2.19)	0.02-0.29
5-15	high	9.8-13.1 (11.5)	0.13-0.40
< 5	low	3.74-13.8 (7.79)	0.16-0.40
< 5	high	2.01-18.2 (7.94)	0.13-0.20

[1]shows range of values and median in parenthesis. PA: phytic acid, PC: phenolic compounds. High PC is classified as having total PC > 1000 mg GAE/100 g and CT. GAE: gallic acid equivalents. Low PC cereals are maize, pearl millet, white sorghum and white finger millet while high PC cereals are red sorghum and red finger millet. Cereals are either fermented or dephytinized enzymatically and may include some additions of baobab fruit pulp or mopane. According to World Health Organization (2004), zinc bioavailability is classified according to the level of the PA as follows: PA > 15: 15%, PA between 5-15: 30% and PA < 5: 50%.

Table 8.4: Bioavailability of zinc from available human studies where cereals where used as test meals

Product description	PA/Zn	PC	% bioavailability	Amount absorbed	Reference
[1]Pearl millet porridge	5-15	low	9.5	0.13 mg/meal	Brnić et al. (2017)
[1]Pearl millet porridge	< 5	low	16	0.22 mg/meal	Brnić et al. (2017)
[1]Maize porridge	> 15	low	8.7	0.24 mg/meal	Brnić et al. (2014)
[1]Maize, white sorghum porridge	< 5	low	15.0-16.5	0.42-.0.46 mg/meal	Brnić et al. (2014)
[1]Brown sorghum porridge	< 5	high	16.6	0.46 mg/meal	Brnić et al. (2014)
[1]White sorghum porridge	> 15	low	10.7	0.32 mg/meal	Brnić et al. (2014)
[1]brown sorghum porridge	> 15	high	8.4	0.24 mg/meal	Brnić et al. (2014)
[1]Maize-soya ready to eat porridge	5-15	low	8.8-10.7	0.13-0.16 mg/meal	Hettiarachchi et al. (2010)
[2]Maize diet	> 15	low	24	1.30 mg/day	Manary et al. (2002)
[2]Maize porridge (biofortified, whole grains)	> 15	low	22	1.1 mg/day	Chomba et al. (2015)
[2]Maize porridge Whole grains	> 15	low	28	0.6 mg/day	Chomba et al. (2015)
Pearl millet porridge (biofortified)	5-15	low	17	1.0 mg/day	Kodkany et al. (2013)
Pearl millet porridge	> 15	low	20	0.7 mg/day	Kodkany et al. (2013)

[1] meals based on decorticated grains, [2] meals based on whole grains. Only human studies where maize, sorghum and millets were used as test meals were considered. Other cereals such as rice and wheat were not considered. All the human studies were based on extrinsic radioisotope studies done mainly on African and Asian children aged between 1-7 years with a high PA background diet. Only one study by Brnić et al,. 2014 was based on European adult men and women.

In one study, phytase was added at the point of consumption of a pearl millet porridge and zinc bioavailability was increased by 68% compared to the control. However, the bioavailability of the dephytinized pearl millet porridge was 16% even though the PA/Zn ratio was < 5 (Brnić et al., 2017). Other porridges prepared from sorghum with PA/Zn close to 0 because of complete dephytinization also had zinc bioavailability close to 15%. From the human studies in **Table 8.4**, it seems that meals based on decorticated cereals with PA/Zn > 5 have a zinc bioavailability of less than 10% while those with PA/Zn < 5 have bioavailability greater than 10% but not higher than 20%. On the contrary, higher zinc bioavailability (22-28%) was observed for high PA maize diets that were based on whole grain maize. Also higher bioavailability of 17-20% was observed in the study of Kodkany et al. (2013) where high PA pearl millet was used but it is not mentioned whether the pearl millet grains were decorticated or not.

These studies show inconsistencies on the effect of PA on zinc bioavailability. Our findings suggest that dephytinization of whole grains does not necessarily increase the zinc bioaccessibility and that the effect of PA on zinc bioaccessibility may be evident in some cases in particular for maize (chapter 6) but does not appear to be dose dependent. This could be because in decorticated grains, the bran layer which contains the most inhibitors, is removed and PA in the endosperm becomes the most important inhibitor such that dephytinization causes a significant improvement on zinc bioavailability. On the other hand, presence of bran in whole grain cereals means that zinc is still bound to other inhibitors such as PC which are highly associated with the cell walls and the removal of PA without the removal of PC may not increase the bioavailability significantly. Nonetheless, the effect of PA was more evident on zinc bioaccessibility than on iron bioaccessibility. Concerning the effect of PC and CT on zinc bioaccessibility, high PC cereals, in particular red sorghum, had lower zinc bioaccessibility than low PC cereals but this effect was not consistent especially for red finger millet which had comparable zinc bioaccessibility with other low PC cereals like maize and white finger millet. Brnić et al. (2014) showed that the effect of PC on zinc bioavailability in red sorghum was only apparent in the presence of PA and this agrees with our findings on fermented red sorghum (Chapter 4 and 7) but not on the enzyme treated red sorghum (Chapter 6).

What is noteworthy in the human studies is the magnitude of response which is comparable to our bioaccessibility results. Results from human studies ranged from 8.4-28% while our results (Chapter 3, 4, 6 and 7) ranged from 0.45-25.9% (median 9.21%) with PA/Zn values ranging from high (> 15) to low (< 5). Lower zinc bioaccessibility values in the vicinity of 1% may have been obtained in our study due to effect of other factors that are unknown such as the effect of cereal matrix, the limitation presented by dialyzability assay and also because the human studies were based on complete meals. Studies using specific test meals to validate *in vitro* studies against *in vivo* studies are urgently needed.

Current estimations from World Health Organization (2004) on zinc bioavailability need to be revised as zinc bioavailability of 30% is expected when PA/Zn is between 5-15 and even 50% when there is complete dephytinization but this was not the case in both our results and the human studies. In fact, diets based on fermented cereals are estimated to have moderate zinc bioavailability (30%) (World Health Organization, 2004). It is generally accepted that the estimates on zinc bioavailability based on PA/Zn are only permissible when there are no other inhibitors, especially PC, present but this was still not the case when considering low PC cereals such as maize, white sorghum and white finger millet. Estimations of zinc bioavailability from dietary intake and food composition tables thus need to be revisited as these values give misleading information especially when estimating country prevalence data. A study by Miller et al. (2015) revealed the absence of PA effect on zinc absorption in children and that PA reduction as a strategy to improve zinc nutrition might not be the solution. The amount of zinc present in the cereal may be more important than the bioavailability as some studies using biofortified maize and pearl millet have exemplified. Biofortified cereals provided more absorbable zinc than non-biofortified cereals despite high PA levels and could meet absolute requirements for children (Chomba et al., 2015; Kodkany et al., 2013). Programs aimed at improving bioavailability of cereals should thus focus on significant results that actually have a tangible effect on zinc nutrition.

In terms of *in vitro* methodologies for estimating iron and zinc bioavailability, there is need for a consensus on the methods that closely predict bioavailability. In our study, we have used a consensus method that has been standardized by a network of researchers working in the field of food digestion (Minekus et al., 2014). The adoption of this method by other workers researching on mineral bioavailability may allow for better comparison of results. In addition, there is a need for *in vitro* digestion models for different age groups in particular for children as some studies have indicated the differences in the absorption of zinc for children and adults. Higher absorption of zinc from high PA diets in children than in adults has been attributed to the action of dietary microbial phytases which could be active during gastric digestion because of the high pH in children of between 4-6, optimal for acid phytases (Miller et al., 2015). Many human studies on iron and zinc using cereals as test meals have been conducted on adults, at the same time estimations on iron and zinc bioavailability using PA/Zn and PA/Fe have been based on adult absorption studies and it is still not clear if this can be applied to children as well.

8.2 Effect of fermentation on mineral binders and subsequent iron and zinc bioaccessibility

The goal of this PhD research was to evaluate the efficacy of fermentation as a food based strategy to improve iron and zinc bioaccessibility of traditional fermented porridges from Zimbabwe. In this

section, the effect of fermentation on the bacterial communities and mineral binders PA, PC and CT will be discussed, as well as on the subsequent iron and zinc bioaccessibility of cereals.

8.2.1 Is there a relationship between bacterial communities and iron and zinc bioaccessibility?

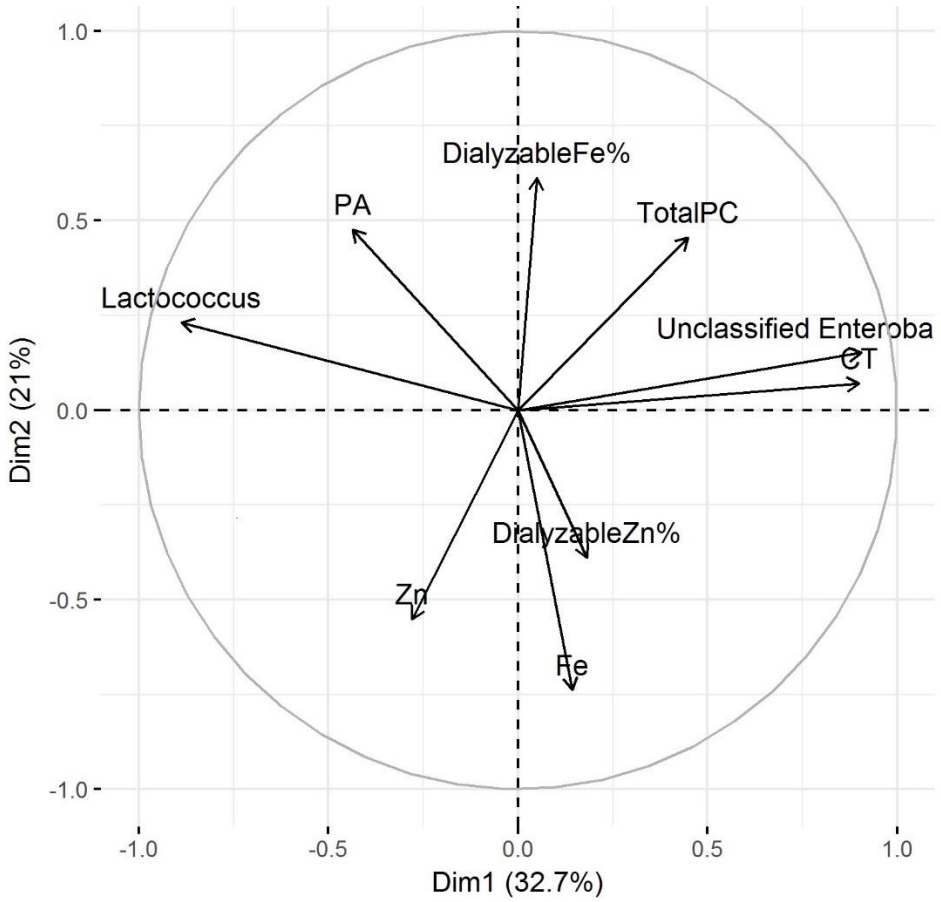
In chapter 5 of this PhD, the presence of LAB from *Lactococcus*, *Weissella*, *Leuconostoc* and *Enterococcus* was established in both household and laboratory cereal fermented slurries. In addition, unclassified *Enterobacteriaceae* and some Proteobacteria were also detected. To determine if there was a relationship among bacterial communities, mineral binders and iron and zinc bioaccessibility, principal component analysis (PCA) was done using the following parameters observed in laboratory fermented slurries: PA, PC, CT, total iron and zinc, bioaccessible (dialyzable) iron and zinc and the %abundance of *Lactococcus* and unclassified *Enterobacteriaceae*. *Lactococcus* and unclassified *Enterobacteriaceae* were the dominant genera in laboratory fermented slurries. **Figure 8.1A** shows the results of the PCA. About 53.7% of the variation in laboratory fermented slurries was explained by dimension 1 (32.7%) and dimension 2 (21%). Dimension 1 was related to abundance of unclassified *Enterobacteriaceae* and CT and abundance of *Lactococcus* in the opposite direction. **Figure 8.1B and C** shows the individuals plot which reveals the loading of fermented sorghum from Chiweshe and Hwedza (red sorghum samples – group 1) on the upper right quadrant of the plot along dimension 1 with abundance of unclassified *Enterobacteriaceae* and CT. Indeed, fermented red sorghum had high abundance of unclassified *Enterobacteriaceae* and CT. In chapter 5, the presence of a higher abundance of unclassified *Enterobacteriaceae* in red sorghum than in other cereals was discussed and it was concluded that the CT in red sorghum may have some antimicrobial effect on the *Lactococcus* which were abundant in other cereals. Although the risk of the presence of unclassified *Enterobacteriaceae* was highlighted, the fact that they outcompeted other LAB in the CT environment of red sorghum means that they are tolerant to this environment and could in fact, play a functional role at least at that time point of the fermentation (26 h). *Enterobacteriaceae* have also been identified after 30 h of cocoa bean fermentation and it was suggested that they could have a functional role in citrate assimilation and carbohydrate metabolism (Illeghems et al., 2015).

Phytic acid reduction was observed in both red sorghum (22-44%) and other cereals (20-88%) suggesting the presence of phytase producing microbes in all matrices. To our knowledge, no *Lactococcus* or *Enterobacteriaceae* have been isolated from African fermented gruels and tested for phytase activity. These studies have only been confined to *Lactobacillus* and *Lactobacillus* with phytase activity has been observed in ben-saalga (Songre-Ouattara et al., 2008). In addition, the ability to metabolize PC has also been focused on *Lactobacillus* species and not on other LAB, and certainly not

on *Enterobacteriaceae*. Since other LAB such as *Lactococcus*, *Weissella* and *Leuconostoc* seem to play an important role on the fermentation of maize, sorghum and millets, future studies should focus on their functional role. Moreover, the functional role of *Enterobacteriaceae* in matrices with CT needs to be elucidated to better understand the microbial dynamics under these conditions.

Dimension 2 on the other hand, was related to the %dialyzable or bioaccessible iron, PC and PA in one end and in the opposite direction was dialyzable or bioaccessible zinc and total iron and zinc content of the fermented slurries. Three groups can be observed from the individuals plots (**Figure 8.1B and C**). Group 1 shows red sorghum samples from Chiweshe and Hwedza with high abundance of unclassified *Enterobacteriaceae* as explained before, group 2 samples includes samples from all locations except all Chiredzi samples and also all cereal grain types except red sorghum and most of the finger millet. Samples from group 2 show basically the generality of the laboratory fermented slurries which was characterized by abundance of *Lactococcus*, high PA, moderate bioaccessible iron and generally low bioaccessible zinc. Group 3 samples on the other hand, shows all samples from Chiredzi which were contaminated by extrinsic iron and also all finger millet samples except finger millet from Chiweshe which loaded on the upper middle part of the quadrant (**figure 8.1B and C**) because it had high bioaccessible iron. These samples were characterized mainly by high iron content (in particular Chiredzi samples) and low bioaccessible iron.

Bacterial communities do have a relationship with mineral binders in particular PC and CT although the subsequent effect on iron and zinc bioaccessibility was not conclusive. The correlation between abundance of unclassified *Enterobacteriaceae* and total PC and CT was 0.812 and 0.842 respectively, while the abundance of *Lactococcus* and total PC and CT was -0.737 and -0.793 respectively. Since the changes on the PC and CT were marginal, the direct effect of the bacterial communities on the iron and zinc bioaccessibility was not clear. In addition, although phytase activity was evident in the fermented slurries, the relationship between PA and iron and zinc bioaccessibility was not the same for all fermented cereals. Longer fermentation times of at least 4 days have shown the dominance of *Lactobacillus* genera (De Vuyst et al., 2014) and this may be needed to have a more pronounced effect on PC and CT since some species from *Lactobacillus* have been found to have ability to metabolize PC and CT (Svensson et al., 2010). African cereal fermentations are also characterized by microorganisms from yeasts with functional roles such as phytase activity (Hellström et al., 2015; Hellström et al., 2010). A comprehensive characterization of the microbiota of fermented maize, sorghum and millets will thus be beneficial for the production of standardized, safe and nutritious fermented products.



A

Figure 8.1A: Principal component analysis showing variables plot (A)

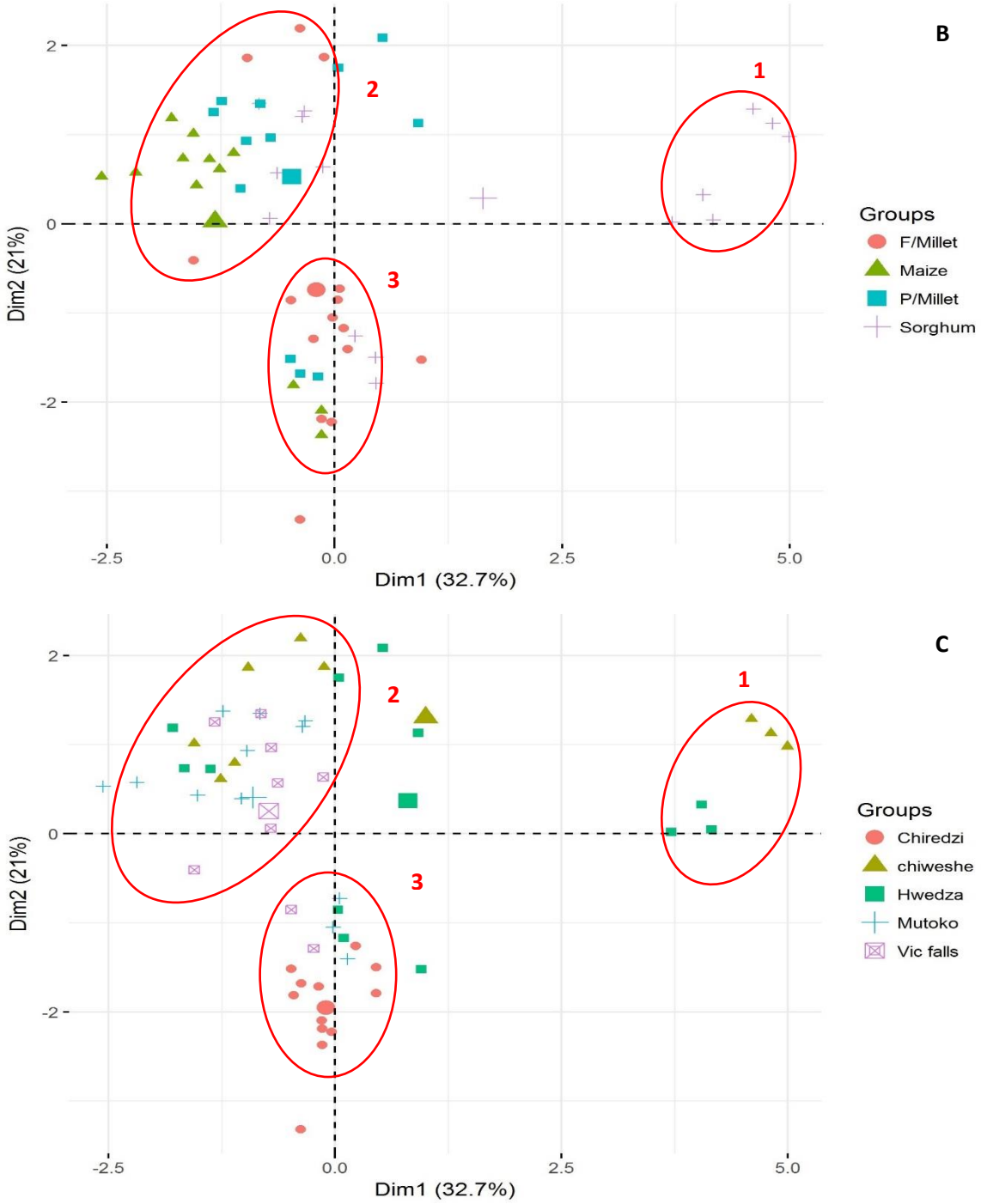


Figure 8.1B and C: Principal component analysis showing individuals plot according to cereal type (B) and according to location (C)

8.2.2 Effect of fermentation on phytic acid, phenolic compounds and condensed tannins

Fermentation caused a reduction of 22-54% PA only in one variety of household fermented finger millet while no reduction in PA was observed in other varieties (Chapter 3). On the other hand, a reduction of 20-88% PA was observed in all cereals that were prepared under laboratory conditions (Chapter 4). The lack of PA reduction in most of the fermented cereals at household level revealed the unpredictability and lack of success of some fermentations performed at household level and underscores the need for optimization of household fermentations. Household fermentations lack in particular temperature and pH control, parameters which could be important for the production of bacterial phytases. During household preparations, temperature is not constant as during the day it is hot with temperatures between 25 and 30^{o}C, while during the night lower temperatures are experienced. Additionally, the performance of fermentation under unhygienic conditions could encourage the proliferation of undesirable microorganisms which do not produce phytases or with phytases that were not active at the conditions of the fermentation. For example, the optimum phytase activity of *L. sanfranciscensis* isolated from wheat was at pH 4.0 and 45^{o}C (De Angelis et al., 2003), so it is conceivable that some phytases might have been produced but the temperature was probably not optimal. Also the pH may not be optimum as some bacterial phytases such as those produced by bacteria from the genera *Enterobacter* operate optimally at pH 6-8 (Jorquera et al., 2008). Although the same microbial communities were found in both household and laboratory fermented slurries (Chapter 6), differences at species level could result in the presence or absence of phytase activity. The unpredictability and risks associated with household fermentations underscores the need for educating communities about hygiene and basic concepts of fermentation. Fermented foods should have a pH of less than 4.0 (Nout, 2009) in order to guarantee microbial safety but the fermented cereals in this PhD study (chapter 3 and 4) had a pH between 4 and 5, and some even above 5 (chapter 4). In hindsight, this may be considered as not important since the fermented cereals will undergo a cooking step, but as mentioned in chapter 5, cooking may not guarantee destruction of toxins that may be produced by bacteria such as *Pseudomonas*. It is therefore important that the culture of conducting the fermentation in a correct way is inculcated into communities.

Nonetheless, our study has shown that PA is indeed reduced after fermentation and the magnitude of reduction depends on the cereal matrix and the fermentation environment. It was clear that the PA degradation in maize, sorghum and millets is most likely coming from microbial phytases and not due to the activation of endogenous phytases at low pH (Chapter 4) since in the household fermentations (Chapter 3), pH was reduced from 4.0-4.5 but still no reduction in PA was observed. Most cereal endogenous phytases are activated at pH 4.5-5.0 (Anastasio et al., 2010) but endogenous phytases

may be unimportant in the type of cereals studied in this PhD because their levels are generally low and are mostly activated by germination (Brinch-Pedersen et al., 2014).

For both household and laboratory fermented cereals, the changes on PC occurred mostly on the soluble PC which are readily leached into the fermentation water while marginal changes occurred on the bound PC. LAB do not possess cell wall degrading enzymes and this characteristic is shown in the lack of release of bound PC. Since the bound PC accounted for the greater part of the total PC, the changes on the total PC were minimal. Several studies have reported on the changes occurring to the PC during fermentation but most of these studies only focus on the soluble PC and not on the bound PC. Bound PC constitute more than 50% of total PC in cereal grains (Acosta-Estrada et al., 2014; Adom and Liu, 2002) and in the cereals studied in this PhD (Chapter 3 and 4), they constituted 64-96% of the total PC. To this end, it can be concluded that a big part of the minerals bound to PC will be associated mostly with the bound PC. The changes to the soluble PC could be attributed to chemical changes i.e. pH effects and also microbial effects as discussed in chapter 3. The effect of fermentation on total PC and CT as it is marginal, is likely not to have a big impact on the iron and zinc bioaccessibility. In addition, relationship between bacterial communities and mineral bioaccessibility was inconclusive because bacterial communities were highly associated with PC and CT.

8.2.3 Effect of fermentation on iron and zinc bioaccessibility

The generally accepted narrative in the scientific community is that the reduction of the mineral binders PA and PC will lead to improved mineral bioaccessibility. The reduction of PA during fermentation in our study did not necessitate a consistent improvement in iron and zinc bioaccessibility and neither was a dose dependent relationship between PA and iron and zinc bioaccessibility observed. One may contend that probably the reduction of PA was not adequate since there was still some residual PA in the fermented cereals and moreover, the PA/Fe and PA/Zn still remained mostly above the critical limits beyond which iron and zinc bioavailability is seriously impaired i.e. PA/Fe > 1 and PA/Zn > 10-15 (Hurrell and Egli, 2010; Saha et al., 1994). However, the enzymatic study (chapter 6) where there was complete dephytinization proved that PA degradation does not induce a consistent improvement in iron and zinc bioaccessibility in whole fermented cereals and also that a reduction in both PA and PC is probably warranted. Indeed PA can be reduced to some extent through fermentation and through enzymatic means as shown in our study and in many other studies (Baye et al., 2015; Baye et al., 2013; Lestienne et al., 2005b), but how can PC be reduced and to what extent should they be reduced? Secondly, is it of absolute necessity to reduce PC and PA to have an increased iron and zinc bioavailability? Dietary fibers seem to be involved as well in the

complexation of minerals but should these components be reduced as well in order to improve the bioavailability of iron and zinc in cereals?

Some degradation of PA may be beneficial for the release of iron bound to PA as shown in the case of maize and for zinc bioaccessibility as shown in the enzymatic study (Chapter 6). Complete dephytinization may not be the solution since some studies have shown that iron bound to PA does not represent an irreversibly unabsorbable form of iron but depending on other parameters in the food matrix, it is potentially bioavailable. An *in vitro* study conducted in the absence of food showed that iron bound to PA was soluble with limited bioavailability which was reduced as PA/Fe increased and this was attributed to the molar excess of PA preventing interaction of iron with iron transporters at the brush border surface (Engle-Stone et al., 2005). Maximal inhibition of iron absorption occurred at PA/Fe ratio of 5 when iron was introduced in the form of $FeCl_3$ and 10 when it was in form of $FeSO_4$ (Engle-Stone et al., 2005; Glahn et al., 2002). Indeed, PA forms complexes with higher stability constants with trivalent metal ions than with divalent metal ions (Torres et al., 2008) as such processes that cause more reduction of Fe^{3+} to Fe^{2+} have positive effects on iron bioavailability. A human study in which dephytinized wheat bran and non-dephytinized wheat bran were used as test meals showed that the group consuming the non-dephytinized wheat bran were in higher positive iron balance compared to the group consuming the dephytinized wheat bran. The PA/Fe of the non-dephytinized wheat bran was 12 (Morris et al., 1988). Other studies in which PA did not seem to influence the mineral bioavailability had PA/Fe ratios of 5-10 (Cercamondi et al., 2013; Kodkany et al., 2013; Mendoza et al., 1998) and 9-34 for PA/Zn (Chomba et al., 2015; Kodkany et al., 2013).

An important factor to also consider is the differences in the mineral binding capacity of different isomers of PA and the lack of information pertaining to the type of PA: mineral isomers existing in different cereals. For example, PA bound to iron in wheat is mostly existing as monoferric phytate which is soluble and potentially bioavailable (Eagling et al., 2014; Engle-Stone et al., 2005). On the other hand, some researchers have suggested that zinc is bound to PA in PA-zinc saturated complexes which are insoluble (Eagling et al., 2014). Phytic acid with less than 5 phosphate groups does not impact on zinc bioavailability while PA with less than 3 phosphate groups does not impact on iron bioavailability (Lönnerdal et al., 1989; Sandberg et al., 1999). The inconsistencies related to the effect of PA on iron and zinc bioaccessibility observed in this study could thus be related to the different isomeric forms of phytate-mineral complexes that can exist and their differences in mineral bioaccessibility. Partial reduction of PA may be appropriate as emerging studies have also indicated some health benefits of PA. PA has been maligned for decades because of its mineral binding but it is now known to have positive effects against numerous types of cancers i.e. colon, breast, prostate, pancreatic and blood/bone marrow cancers. The mechanism of action is suggested to be through

antioxidant properties, mineral binding ability, pH reduction and promotion of DNA repair. PA is now also suggested to be able to fight against coronary heart disease, diabetes mellitus and dental caries (Harland and Morris, 1995; Kumar et al., 2010).

Reduction of PC presents many challenges as they are a highly heterogeneous group which makes the identification of an enzyme with a broad substrate specificity a challenge. In our study, laccase which catalyzes the oxidation of mono, di- and tri- hydroxyl PC was used, thereby encompassing a broad range of PC as substrates (Baldrian, 2006; Cercamondi et al., 2014b). However, changes in PC were only observed on the soluble PC and not on the bound PC in our study. A study by Cercamondi et al. (2014b) showed some reduction on soluble PC after using laccase which was not counteracted by improved iron bioavailability. A major challenge in the enzymatic degradation of PC is that the enzymatic degradation products of PC and their mineral binding ability are not known which makes it difficult to focus on degrading PC as a strategy to improve mineral bioavailability. Most importantly, PC in cereals are well known for their undisputed health promoting properties which include prevention and reduction of oxidative stress, anti-hypertensive properties, anti-inflammatory properties, anticarcinogenesis and prevention of cardiovascular diseases (Dykes and Rooney, 2006, 2007; Taylor et al., 2014). In fact, the utilization of sorghum and millets is on the rise because of their phytochemical contents and subsequent functional properties.

Similar to PA and PC, dietary fibers are also considered to have a negative impact on iron and zinc bioavailability but the health promoting effects of dietary fibers have long been appreciated. Some studies have shown the positive effect of decortication on iron and zinc bioaccessibility due to the removal of mineral binders (Lestienne et al., 2007) but the long term consumption of decorticated cereals may not be beneficial for human health. The inclusion of whole grains in the diets is accompanied with reduced risk of chronic diseases such as coronary heart disease, cardiovascular diseases, total cancer, respiratory diseases and diabetes leading to reduced mortality (Aune et al., 2016). Many studies have proven the benefits of dietary fibers and this has provided the impetus to recommend increased intake of whole grains in dietary guidelines. Dietary fibers also play an important role in gut health due to the prebiotic effect of some oligosaccharides whose fermentation in the colon leads to the production of short chain fatty acids which can form soluble complexes with minerals. The short chain fatty acids can subsequently reduce the pH and provide an optimum environment for the reduction of Fe^{3+} to Fe^{2+}, enhance the proliferation of epithelial cells thereby increasing the absorptive surface area in the colon and also stimulate the proliferation of intestinal microbiota with ability to metabolize PA and PC (Amaro López and Camara Martos, 2004; Yeung et al., 2005). Iron colonic absorption has been reported by Yeung et al. (2005) in response to prebiotics but the efficiency of the colon as a site for iron absorption and the possible impact on iron status still remains a question. Zinc

colonic absorption has also been reported in animal studies as a result of fermentable carbohydrates, but the possibility of colonic absorption and quantitative relevance in humans has to be substantiated (Gopalsamy et al., 2015).

Other mineral binders commonly associated with plant based products include calcium and oxalic acid. Calcium retards mineral absorption, particularly zinc, through the formation of insoluble calcium-zinc-PA complexes. However, the negative effect of calcium was not observed in the present PhD because finger millet contains ten times more calcium than other cereals (Devi et al., 2011). In fact, zinc bioaccessibility of finger millet was mostly higher than other cereals such as pearl millet and sorghum which have lower calcium content. Some studies suggest that the inhibitory effect of calcium on zinc bioavailability is only apparent when calcium is added to the food as a supplement (Jayalakshmi and Platel, 2016; Wood and Zheng, 1997). Oxalic acid is an organic acid found in many plant based products with the ability to bind calcium forming unabsorbable complexes. Oxalic acid has also been reported to have moderate negative consequences towards iron and zinc bioavailability and its effect is higher in high fiber foods whereby there is formation of fiber-oxalic acid-mineral complexes (Noonan and Savage, 1999). The negative consequences of oxalic acid are much higher in vegetables such as spinach where levels of oxalic acid are in the range 976-1198 mg/100 g dm compared to cereals with oxalic content of not more than 100 mg/100 g dm (Gupta et al., 2006; Siener et al., 2006). Moreover, it is suggested that oxalic acid: calcium molar ratios of < 1 do not have a huge impact on mineral absorption and most cereals have oxalic acid: calcium molar ratio of < 1 (Noonan and Savage, 1999). Nonetheless, it may be worthwhile to determine the relationship among all purpoted mineral absorption inhibitors.

In this PhD, the effect of PA, PC and CT on iron and zinc bioaccessibility was not straightforward. Like many other studies, the focus was on improving iron and zinc bioaccessibility through the release of iron and zinc from insoluble complexes of PA, PC and CT. Perhaps, there are other factors that not only includes the effect of mineral binders but also kinetics of release of the iron and zinc from the food, competition for mineral complexation between mineral binders in the soluble and solid phase, and competition between the minerals themselves for complexation. Undoubtedly, studies capturing these aspects concerning iron and zinc bioavailability are crucial.

In light of the health benefits of PA, PC and dietary fibers and their possible negative effects towards iron and zinc bioavailability, it is prudent to suggest that strategies to improve mineral bioavailability have to be considered in the context of complete diets. Suitable compromises have to be found that allow for improved mineral bioavailability at the same time, considering the needs for health promotion and disease risk reduction conferred by PA, PC and dietary fibers. In the next section, some

of the strategies that allow for the retention of PA, PC and dietary fibers will be discussed, and yet have potential to change the trajectory of mineral nutrition in developing countries.

8.3 Towards an improvement in mineral nutrition through a multidisciplinary approach

We have established that to improve iron and zinc bioavailability, it may not necessarily require a drastic reduction of mineral binders. A possible strategy is the addition of mineral absorption enhancers through food-to-food fortification as exemplified in chapter 7. Through this approach, an increase in iron and zinc bioaccessibility after adding baobab fruit pulp was observed. This result was attributed to enhancing components in baobab fruit pulp most likely ascorbic acid and some small organic acids. Several other studies have shown improved iron and zinc bioaaccessibility in cereals after adding food sources with mineral enhancing compounds despite high PA and PC in the cereals (Gautam et al., 2010a, b, 2011; Hemalatha et al., 2005; Kumari and Platel, 2016). The enhancing components were derived from high sulphur spices such as garlic and onion, organic acids from amchur (*Mangifera Indica*) and citric acid and β-carotene from β-carotene rich vegetables such as carrots. Food-to-food fortification is not only beneficial for the improvement of mineral bioavailability but also for the increase in mineral contents which are inherently low in some cereals. The addition of insects such as mopane worm with higher iron and zinc contents than the individual cereals may provide more absorbed iron and zinc from enriched cereals than the non-enriched cereals as shown in chapter 7. Addition of insects through food-to-food fortification holds much potential, as not only do insects contain high iron and zinc contents, but insects such as snails (*Limicolaria sp.*) and termites (*Achatina fulica, A. marginata, A. achatina*) also contain haem iron which is more bioavailable than non-haem iron (Chadare et al., 2017). These insects are highy accessible to many poor communities in developing countries. An interesting opportunity presented by insects as a vehicle for food-to-food fortification is also their high protein content. Protein hydrolysates (amino acids and peptides) may be important as enhancers of non-haem iron bioavailability and some of the potent amino acids in iron-chelation such as glycine, histidine, serine and cystein have been identified in some insects (Li et al., 2017; Nongonierma and FitzGerald, 2017).

Partial reduction of PA by fermentation followed by food-to-food fortification may thus allow for improved mineral nutrition in developing countries. Food-to-food fortification is a strategy that is immediately available to vulnerable populations and represents a resource effective and sustainable approach to addressing mineral deficiencies in these areas. Future studies should focus on providing a thorough inventory of the nutritional composition of traditional food sources in order to carve out the potential of certain food sources to improve mineral nutrition. A recent study by Chadare et al. (2017)

investigated the nutritional composition of indigenous food ingredients from Benin in order to evaluate their potential for utilization in complementary food formulations to combat malnutrition in infants. Several indigenous foods such as *Moringa oleifera* leaves, baobab fruit pulp pulp and leaves, seeds and pulp of *Parkia biglobosa*, were found to have high ascorbic acid content and high iron and zinc content thereby providing many opportunities for the innovation of complementary porridges of high nutritional value which can meet the iron and zinc requirements of children. This study can be used in other African countries where the same foods can be found but undoubtedly, more studies of this kind are required to cover all indigenous food sources in the region. Food-to-food fortication should not just focus on enhancing mineral contents and bioavailability but should perhaps be considered in the context of a balanced meal. In particular the energy density of the porridges should be considered. In chapter 3, it was observed that porridges without peanut butter had a low energy density which could not meet the requirements for complementary porridges and the same trend was also observed for porridges from other African countries. In this regard, indigenous food ingredients that can balance both energy density and mineral contents will be particularly useful. The need for the production of nutritious complementary porridges in Zimbabwe is highly critical as the traditional porridges discussed in this PhD are common in most households.

Food science and technology is needed in the fight against mineral malnutrition but food science and technology alone cannot alleviate this problem. Minerals in plant foods originate from soil. It has been shown that the mineral status of soils in a region is reflective of the mineral status of the population and this is particularly true for iodine which is occurring in low amounts in soils from inland countries and was a cause of concern before salt iodization was introduced (Bevis, 2015). The flow of minerals in the food system is thus from soil-crop-processed product-human. This mineral transmission process is important to understand as it provides several entry points for intervention and demonstrates the need for a mulitdisciplinary approach to eradicate mineral deficiencies.

In this PhD, zinc contents of cereals were generally low ranging from 0.94-4.39 mg/100 g dm. A typical Zimbabwean complementary diet consist of cereal based meals administered three times a day and includes the thin porridges discussed in this PhD which can be consumed maximum twice a day (dm 20-30%) (chapter 2) and a thick porridge which is normally consumed as an evening meal and sometimes also for lunch (dm 30-40%). The absolute zinc bioavailability can be 5% and according to the results from this study and from the human studies; zinc bioavailability may likely not exceed 15% in many cereals. If we consider a conservative dm of 30% and uppper bioavailability of 15% and consider that complementary porridges should provide at least 75% of the absolute zinc requirements (1.2 mg/day for children aged 1-3 years), a 100 g meal should be able to provide at least 0.3 mg/meal.

This means that the zinc content should be at least 6 mg/100 g dm in cereals which is lower than the zinc contents determined in this study. In fact, for bioavailability of less than 15%, even higher zinc contents will be needed which may not be attainable.

In this light, zinc contents in cereals can be improved at the soil level by application of zinc enriched fertilizers and zinc foliar. This has been found to be successful in many African countries and increased zinc contents of maize, rice and wheat by up to 63% have been reported (Joy et al., 2015b). In additon, Joy et al. (2015a) further reported that soil types influences mineral composition in plant foods and indicated that zinc contents are higher in calcerous soils than in non-calcerous soils. Indeed, Zimbabwean soils are largely non-calcerous and generally known to be zinc deficient such that soil level interventions are urgently needed. A recent study by Manzeke et al. (2014) showed that zinc fertilization of Zimbabwean maize staple increased the grain zinc content implying that adoption of such agronomic approach nationwide should be promulgated as many rural people rely on the crop produced on their land. Henceforth, the biofortication of cereals is important as it ensures increased zinc contents which can be sufficient to meet zinc requirements at 17-22% bioavailability (Chomba et al., 2015; Kodkany et al., 2013). Obviously, a combination of these strategies together with the food based strategies discussed in this PhD could improve the mineral nutrition of children in vulnerable areas. In this PhD, we have shown that the deficiency risk of zinc could be much higher than that of iron based on the low zinc contents in cereals and also the low zinc bioaccessibility. This is exacerbated by the fact that absolute requirements for zinc are twice that of iron and yet zinc in cereals is occurring at much lower levels than iron. The higher risk of zinc deficieny than iron in Africa has also been shown by other authors and reveals the need for more strategies to target zinc in mineral intervention (Joy et al., 2015a; Siyame et al., 2013).

Unlike zinc, the soil iron status does not necessarily reflect the iron status of the population as estimates based on the dietary intake data and food composition tables is often not corroborated by the high rates of anemia (Joy et al., 2015a). Of course, anemia can occur without iron deficiency but it is generally used as a proxy of iron deficiency as where anemia is prevalent, iron deficiency is considered as the primary cause and up to 50% of the anemia cases are estimated to be caused by iron deficiency (Gregory et al., 2017; Stoltzfus, 2003). Iron levels in soils are high because of the abundance of iron in the earth and can be as high as 40 000 mg/kg compared to 50 mg/kg for zinc in soils (Joy et al., 2015a). The available iron content of the soils from the five locations studied in chapter 4 were above the recommended level of 5 mg/kg (Nikolic et al., 2016) and ranged between 30-141 mg/kg (data not shown) showing that soil iron content may not be the limiting factor in iron nutrition. The iron contents of the cereals ranged between 0.78-49.7 mg/kg dm clearly demonstrating that in some

crops, there could be issues pertaining to low soil-crop iron transmission while in some crops such as in pearl millet, transmission of iron from the soil to the crop seems to be efficient. Iron and/or zinc efficiency in crops is a genetic trait whereby some genotypes are able to efficiently use iron and/or zinc in iron/zinc stress conditions and the mechanism of this process can be through; root processes and architecture that increase the bioavailability of soil iron/zinc for root uptake, enhanced root uptake and translocation and more efficient biochemical utilization of the iron/zinc (Clárk, 1983; Hacisalihoglu and Kochian, 2003).

Using the same caveats as we used for zinc, we can estimate that at least 5 mg/100 g dm iron is needed in cereals assuming 10% bioavailability and 10 mg/100 g assuming 5% bioavailability. Iron contents of 5-10 mg/100 g dm are quite attainable as some cereals such as pearl millet used in this PhD had iron contents > 5 mg/100 g dm. Such levels and even more have also been realized after biofortification of cereals (Cercamondi et al., 2013). It has to be mentioned also that because of the relative abundance of iron in the environment, contamination of cereals by soil iron is very common as observed in the soil contaminated cereals from Chiredzi and Chiweshe in chapter 4. We have also seen in chapter 4 that the contribution of iron to the RDA of children aged between 1-3 years can be as high as 400% and iron requirements can be met by soil contaminated cereals even at the lowest bioavailability of 1%. In some cereals such as finger millet and maize, there are plagued with low iron contents despite being grown on iron sufficient soils hence the biofortification of these cereals to produce high iron varieties is crucial. Biofortification of pearl millet with iron caused absorption of sufficient iron that met dietary requirements at 9% bioavailability (Kodkany et al., 2013). In addition, current evidence on iron biofortification of staple crops reveals the efficacy of biofortifation despite high content of mineral binders (Finkelstein et al., 2017). A study by Kruger et al. (2012) showed that fermentation of biofortified sorghum produced more bioaccessible iron than the non-biofortified control. The improvement of mineral contents in cereals is thus important as only small steps in improving bioavailability will be needed to meet the dietary requirements.

Food based strategies are important to the attainment of optimum mineral nutrition in developing countries but it has to be mentioned that the use of one strategy may not be enough to subvert the consequences of mineral deficiencies. In this PhD we have shown that fermentation may be important to increase iron and zinc bioavailability through partial degradation of PA and reduced pH which provides a buffering capacity during digestion but it is not adequate. We have shown that the combination of fermentation with food-to-food fortification has potential to improve mineral nutrition in low income countries. On the other hand, we have also explored the possibility of combining food based strategies with other approaches not studied in this PhD that are based on soil and crop factors

and these approaches should be considered where applicable. In **Figure 8.2** we show the transmission of minerals from soil to human and possible strategies that can be implemented at each level indicating the need for mulitidisciplinary collaboration.

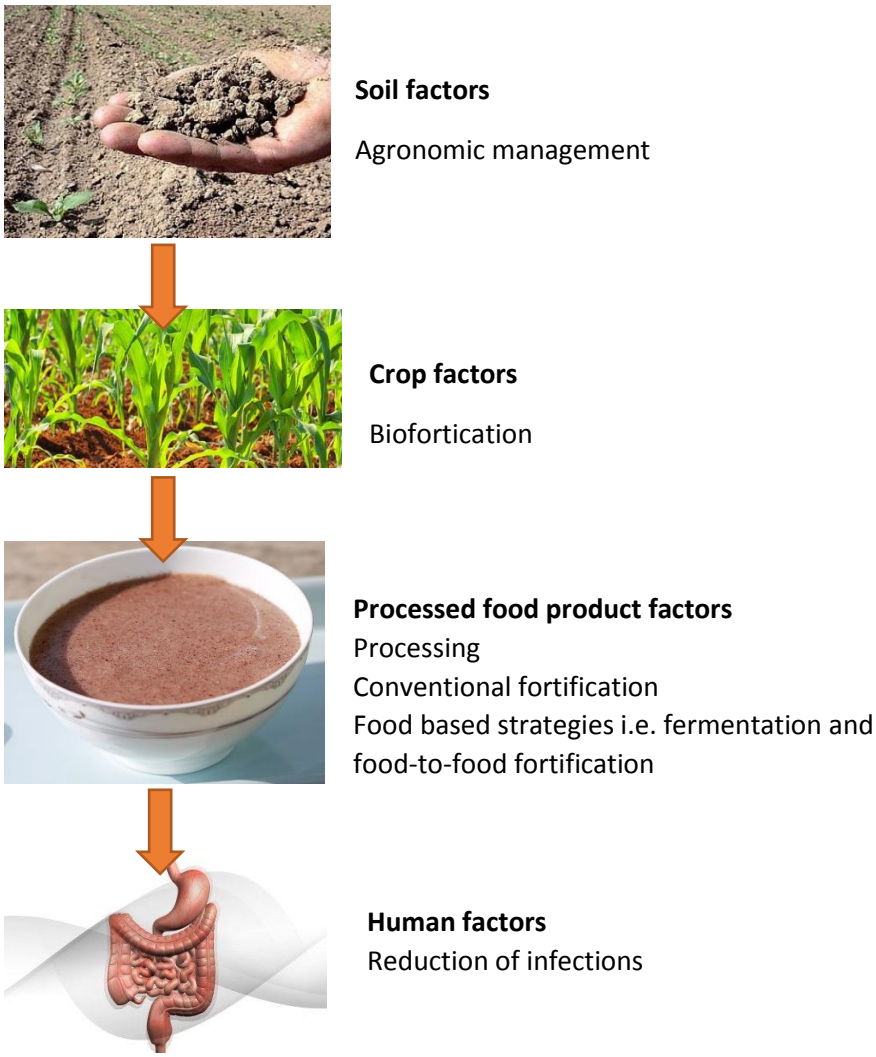


Figure 8.2: Schematic outlining the various entry points for the alleviation of iron and zinc deficiencies

Currently, the Zimbabwean government has adopted a food fortification program which will foresee the fortification of maize flour with iron and zinc along with other micronutrients. This program will improve the mineral nutrition of the urban population whereas for the rural population who are reliant on agricultural activities and process their own cereals, it is important for the government to consider the soil-crop factors and food based strategies. It is noteworthy that the rural population is also the most vulnerable to mineral deficiencies.

8.4 Other minerals of importance in low income settings

In the beginning of this PhD, the major issue underlying the high iron and zinc deficiencies in low income settings which is the reliance on cereal staples for energy and micronutrient intake was discussed. This phenomenon means that there are high chances of multiple mineral deficiencies as most minerals have to be obtained from cereals. **Figure 8.3** shows the contribution of the major food groups to the dietary supply of some of the minerals of importance in Africa. From **Figure 8.3** it is clear that more than 50% of the intake of some minerals is derived from cereals. According to Joy et al. (2014), the risk of deficiency is highest for calcium (54%), followed by zinc (40%), selenium (28%), iodine (19%), iron (5%), copper (1.1%) and magnesium (0.7%) with some regions having even much higher risk of deficiencies. Other minerals of concern in developing countries besides iron and zinc are thus calcium, selenium and iodine whose deficiency risk is very high. This also suggests that the risk of multiple mineral deficiencies in populations is high for example, in Ethiopia, risk of calcium deficiency was 100%, iodine (64%), selenium (36%), zinc (81%) while for Malawi the risk of calcium deficiency was 61%, iodine (27%), selenium (64%) and zinc (33%) (Joy et al., 2014).

One of the possible reasons for high calcium and zinc deficiency is the formation of calcium-PA-zinc complexes and the apparent effect of calcium on zinc bioavailability when calcium is added to food as a supplement, or when it is not in the same food matrix as zinc. Indeed, according to **Figure 8.3**, dietary calcium intake is mainly contributed from animal products most likely dairy products, as such the effect of calcium, zinc and PA may be evident in the context of a complete diet. Where there is zinc deficiency there is likely to be selenium deficiency because both minerals exist in lower quantities in non-calcareous soils than in calcareous soils. As mentioned before, Zimbabwean soils are highly non-calcareous and generally zinc deficient and this indicates the likely presence of selenium deficiency as well (Joy et al., 2015a). The existence of multiple deficiencies of calcium, zinc and selenium is high such that future studies should consider their interrelationship and improve mineral nutrition in cereal based products without considering only an individual mineral but multiple minerals that could possibly be resolved using the same strategies. For example, the innovation of complementary foods with high mineral content can include food sources such as finger millet (high calcium), pearl millet (high iron and zinc), leafy vegetables (high zinc), baobab fruit pulp (high ascorbic acid and organic acids), *Moringa oleifera* leaves (high selenium) (Joy et al., 2015a). The improvement of the mineral content of cereal products thus has to be visualized in the context of all minerals that might be deficient in the population.

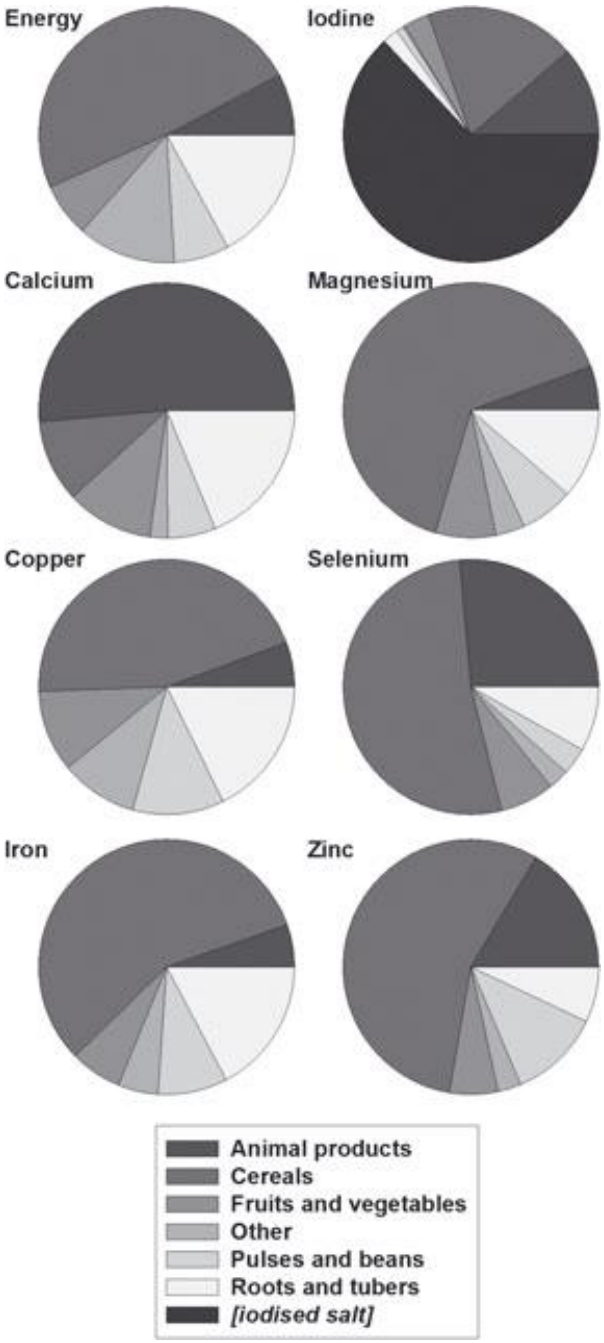


Figure 8.3: Contribution of the major food groups to mineral dietary intake in Africa
Adapted from: (Joy et al., 2014)

8.5 Future research recommendations

A summary of the future research recommendations identified in this PhD are as follows:

- The impact of consuming fermented cereals on iron and zinc nutrition needs to be evaluated in-vivo;
- Inadvertent ingestion of soil iron through cereals contaminated by soil iron is common; as such the impact of soil iron on human iron nutrition needs to be assessed;

- There is a need for more *in vivo* studies that demonstrate the effect of age and race (e.g. difference between studies done on African and European subjects) on iron and zinc absorption;
- As *in vivo* studies are expensive, there is a need for the design of *in vitro* digestion models that better simulate *in vivo* conditions and provide better estimations of bioavailability
- The inconsistencies of PA: mineral molar ratios in estimating iron and zinc bioavailability need to be revisited;
- The complex redox reactions of iron during digestion affect its bioavailability and needs to be understood through mechanistic studies that can provide information on the changes in redox reactions of iron at each stage of digestion and their subsequent effect on iron bioavailability;
- There is a paucity of information concerning the kinetics of release of iron and zinc from different foods during digestion and innovation in the design of experiments is needed to understand this;
- The use of different digestion models makes comparison of results challenging such that there is an urgent need for the harmonization and adoption of universal and consensus digestion models to study iron and zinc bioaccessibility;
- There are many parameters influencing iron and zinc bioavailability i.e. PA, PC, CT, dietary fibers, calcium and oxalic acid. The relationship among all these parameters that have negative consequences on iron and zinc bioavailability in cereals is unclear and needs to be established in the context of complete diets;
- The effect of phenolic compounds on iron and zinc bioavailability in real food systems is unclear since many studies have been done using pure phenolic compounds. There is need for studies using phenolic extracts from foods to examine their effect on iron and zinc bioavailability;
- Food-to-food fortification could improve mineral nutrition in developing countries. An inventory of traditional food sources with potential to enhance mineral contents and bioavailability is urgently needed. Subsequently, studies on how underutilized indigenous food ingredients can be incorporated into complementary porridges are crucial;
- Interventions on iron and zinc deficiencies can be done at soil, crop, food product and host level but the soil and crop factors are necessary for sustainability in most developing countries due to high small holder farming activities. The nexus between agriculture and nutrition should thus be strengthened by conducting more research on how certain agricultural practices can influence iron and zinc bioavailability;

- As fermented food products are important in many African countries as complementary foods, a complete and comprehensive microbial characterization (that includes both bacteria and yeasts) together with the functional capacity of the microbial consortia is needed to enable the production of safe and nutritious fermented foods.

8.6 Generalization

In this PhD, we have discussed the effect of fermentation on the bioaccessibility of iron and zinc in cereals normally consumed in Zimbabwe and Africa at large and also the attributes of household fermentation in comparison with a laboratory fermentation. Having gained some understanding on the relative effects of PA, PC and CT on iron and zinc bioaccessibility through an enzymatic study, the opportunity presented by food-to-food fortification as a sustainable approach to improve mineral nutrition in developing countries was assessed and found to be feasible. Finally, a multidisciplinary approach to improve mutiple mineral nutrition is recommended using approaches that have been studied in this PhD and others that have been found to be successful elsewhere.

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Curriculum vitae

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Education

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Peer reviewed scientific publications

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Submitted manuscripts

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Molly Gabaza, Marie Joossens, Margo Cnockaert, Maud Muchuweti, Katleen Raes, Peter Vandamme, (2017). Lactococci dominate the bacterial communities of fermented maize, sorghum and millets in Zimbabwe. International Journal of Food Microbiology. Submitted.

Conference presentations

Molly Gabaza, Katleen Raes, Stefaan de Smet, Erick Claeys. Potential of the use of vegetable nitrate as nitrite source for meat curing. 59th International congress of Meat Science and Technology, Turkey, August 18-23, 2013.

Molly Gabaza, Promise Muleya, Paul Mapfumo, Maud Muchuweti. Optimization of the utilization of finger millet in the face of climate change. Research and Intellectual Expo, Harare, Zimbabwe, September 2-5, 2014.

Molly Gabaza, Maud Muchuweti, Peter Vandamme, Katleen Raes. Energy adequacy of finger millet complementary porridges in Zimbabwe. Belgian Nutrition Society, Brussels, Belgium, April 2, 2015.

Molly Gabaza, Maud Muchuweti, Peter Vandamme, Katleen Raes. Phenolic compounds and trace minerals in fermented finger millet slurries of Zimbabwe. VIth Sourdough Symposium, Nantes, France, September 30-October 2, 2015.

Molly Gabaza, Maud Muchuweti, Peter Vandamme, Katleen Raes. *In vitro* iron and zinc bioaccessibility of fermented finger millet complementary porridge consumed in Zimbabwe. 8th World Congress on Food Science and Technology, Dublin, Ireland. August 21-25, 2016.

Trainings

Functional ingredients for tailored foods. Agrocampus L'Ouest, Rennes, France, March 2012
Project Management (2014, Project Management Institute of Zimbabwe)

Doctoral school trainings

Advanced Academic English: Poster presentation (2013, Ghent University)
Fostering responsible conduct of research (2015, Ghent University)
Leadership Foundations (2015, Ghent University)
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Introduction to R (2017, Ghent University)

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